EVALUATING THE ASSOCIATION OF HORMONAL CONTRACEPTIVE USE WITH HPV DETECTION IN PRE- AND PERIMENOPAUSAL WOMEN IN THE U.S.

by
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ABSTRACT

Introduction:

Long-term and recent use of combined oral contraceptives (COCs) was suggested to be a risk factor of cervical cancer. It is unclear whether the association is driven by COC's effect(s) on carcinogenesis and/or on upstream events of HPV infection, as study findings on the association between hormonal contraceptive use and HPV infection have been inconsistent. Understanding the effect of hormonal contraceptive use on HPV detection and cervical immune milieu in women older than 35 years may provide insight into the biological mechanisms underlying the impact of hormonal contraceptive use on cervical cancer risk.

Objectives:

A prospective cohort of pre- and perimenopausal females (age range: 35-54) who had normal cervical cytology were followed to investigate the association between cervical HPV detection and hormonal contraceptive use (n=530). The study has 3 aims: Aim 1: To determine the association of hormonal contraceptive use with HPV prevalence at baseline. Aim 2: To compare the odds of new HPV DNA detection between users and non-users of hormonal contraceptives. Aim 3: To describe and compare differences in the host cervical cytokine profiles between hormonal contraceptive users and non-users who were HPV-negative at baseline.

Methods:

Pre- and perimenopausal women (age range: 35-54 years) were recruited from gynecologic clinics in Baltimore, MD. Information was collected on hormonal

contraceptive use, HPV genotypes, history of other sexually transmitted infections, Pap smear diagnoses, sexual behavioral and reproductive characteristics. For Aim 1: associations were assessed using prevalence ratios (PRs) with 95% confidence intervals (CIs). For Aim 2: associations between hormonal contraceptive use and incident HPV detection were measured by odds ratios (ORs) with 95% confidence intervals (CIs) estimated in generalized estimating equation models. For Aim 3: baseline pairs of cervical secretion samples matched in 5-year age groups were collected from current and non-current hormonal contraceptive users who were HPV-negative. Extracted specimens from cervical secretion samples were tested for 27 cytokines, chemokines and growth factors. Comparison of individual mean cytokine levels were made between user groups of hormonal contraceptives. Profile comparison was done using Spearman's rank correlation to determine correlations for all pairwise combinations of the cytokines, chemokines, and growth factors measured in different strata of hormonal contraceptive use and type.

Results:

For Aim 1: more than 5 years' use of progestin-only contraceptives (POCs) was associated with 3-fold increased prevalence of any HPV [adjusted prevalence ratio (aPR): 3.16 (95% CI: 1.82-5.48)] and high-risk (HR)-HPV [aPR: 4.26 (95% CI: 1.60-11.30)] as compared to never POC users. Current POC use was positively associated with prevalence of HR-HPV (aPR: 2.44 (0.99-5.99) and any HPV (aPR: 1.58 (0.94-2.65) with the estimates bordering on statistical significance. For Aim 2: Relative to never POC users, increased incident detection of HR-HPV was observed among current users of POCs [adjusted odds ratio (aOR): 3.24 (95% CI: 1.37-7.65) after controlling for sexual

behavioral factors. No similar associations were observed with the duration or recency of overall hormonal contraceptive or oral contraceptive (OC) use. For Aim 3: Compared to current users of hormonal contraceptives, significantly more positive correlations were detected among proinflammatory cytokines as well as between proinflammatory and immunoregulatory cytokines in current hormonal contraceptive users. Compared to COC users, correlation coefficients were significantly lower in magnitude among immunoregulatory cytokines such as IL-9, IL-15 and IL-17, as well as IL-5, IL-15, IL-17 in current POC users. Relative to non-current hormonal contraceptive users, current POC users were also found to have statistically significantly lower levels of IP10 and several immunoregulatory cytokines, including IL-12, IL-13, IL-15.

Conclusions:

Long-term and current use of POCs may be associated with increased risk of prevalent and incident HPV infection independent of sexual behavior among older women with normal cervical cytology. Our laboratory study showed differences in cytokine profiles between current and non-current hormonal contraceptive users as well as between current POC and COC users in HPV-negative women. These findings suggest differential impacts of exogenous hormones on cervical cytokine milieu, which in turn may potentially affect host responses to local genital infections.

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Overview of the Dissertation

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OVERVIEW

This thesis examined the association of hormonal contraceptive use with cervical human papillomavirus (HPV) detection among pre- and perimenopausal women in the U.S. Data were obtained from an observational prospective study, HPV in perimenopause (HIP), conducted at gynecological clinics associated with the Johns Hopkins Medical Institutions in Baltimore, Maryland.

The natural history of HPV during the menopausal transition is poorly understood (1-3). After the age of sexual debut, a second prevalence increase is observed around the age of menopause in several populations (1-3). This prevalence increase has been postulated to relate to changes in sexual behavior, age-related decreased immune responses and/or menopause-related endogenous hormonal changes (1, 4-6). Disruption of female sex hormonal profiles have been suggested to suppress immune responses to HPV and influence cervical cytokine profiles in vitro (7-15). While the influence of hormonal contraceptive use on HPV infection and cervical cancer has been evaluated previously among young women (16-35), its effect remains largely unclear among older women. A more intensive investigation of hormonal contraceptive use on HPV detection is therefore warranted in women approaching or going through menopause.

This chapter serves as an introduction to the specific aims, brief methodologies, as well as public health significance of the studies undertaken in this dissertation. Chapter 2 gives a brief literature review of the natural history of HPV infection, as well as current

knowledge on the role of hormonal contraceptive use in HPV infection and progression. Chapter 3 provides a manuscript on the association between hormonal contraceptive use and the prevalence of HPV detection in pre- and perimenopausal women. Chapter 4 addresses the association of hormonal contraceptive use with new detection of HPV among pre- and perimenopausal women who had normal cervical cytology. Chapter 5 is a manuscript comparing the host cervical cytokine profiles between current and non-current hormonal contraceptive users among HPV-negative women who were aged over 35 years. Chapter 6 summarizes our study findings and discusses the strengths, limitations, public health implications of this dissertation research and future research directions.

SPECIFIC AIMS

The following specific aims were addressed in this thesis:

<u>Aim 1:</u> To determine the association of hormonal contraceptive use with the prevalence of cervical HPV DNA detection

A cross-sectional study was performed using baseline data collected on the recency and duration of overall hormonal contraceptive use, oral contraceptive use, and progestin-only contraceptive use. Days of menstrual cycle, social demographic factors, sexual behavioral factors, reproductive history and smoking status were evaluated as potential confounders for HPV detection. Prevalence ratios with 95% confidence intervals were estimated using generalized linear models with Poisson regression.

Aim 2: To estimate the association between hormonal contraceptive use and incident cervical HPV DNA detection

Associations were evaluated with unadjusted and adjusted odds ratios with 95% confidence intervals using generalized estimating equation models. Primary exposures of interest included the recency and duration of overall hormonal contraceptive use, progestin-only contraceptive use and oral contraceptive use.

Aim 3: To describe and compare differences in the host cervical cytokine profiles between current hormonal contraceptive users and non-current users

A baseline cross-sectional study was performed using pairs of cervical secretion samples collected from current hormonal contraceptive users and non-current users who were matched in 5-year age groups. Subjects included were HPV-negative at baseline. Comparison of 27 individual mean cytokine, chemokine and growth factor levels was made between user groups of hormonal contraceptives. Profile comparison was done using Spearman's rank correlation coefficients to determine correlations for all pairwise combinations of cytokines, chemokines, and growth factors measured in different strata of hormonal contraceptive use and type.

PUBLIC HEALTH SIGNIFICANCE

This research was motivated by the need to better understand how hormonal contraceptive use may impact HPV detection among older women, which is relevant to the application of HPV DNA testing in cervical cancer screening in this population.

HPV DNA testing is anticipated to have increasing utility in early detection of cervical precancerous lesions in women, particularly in the post-vaccine era when cervical disease will become rarer and may escape cytology-based detection (36-42). Primary HPV DNA testing with cytology triage has been shown to be more sensitive than conventional morphology-based screening such as Pap smear (37-41), and more specific than conventional screening to detect high-grade cervical neoplasia in women aged over 35 years (38).

Counseling for HPV-positive, cytology-negative women and optimization of screening intensity among older women can be challenging (41), because current knowledge is limited on (i) the risk factors for prevalent and persistent HPV detection in older women, (ii) whether the impacts of these risk factors are transient or long-term; and (iii) how these risk factors may predict the risk of progression to high-grade cervical lesions.

Data from this dissertation research provide improved understanding of the association between hormonal contraceptive use and HPV detection in older women. The findings will contribute to generation of hypotheses and future studies to better define natural history of HPV infection among older females, which ultimately may benefit future assessment of cervical cancer screening infrastructure in this age group.

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CHAPTER 2:

Introduction

by

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OVERVIEW: HPV AND CERVICAL CANCER

Disease burden of cervical cancer

In the U.S., cervical cancer is the third most common gynecologic cancer in women (1). The overall incidence of cervical cancer is 7.5 cases per 100,000 women in the U.S. (2). Worldwide, cervical cancer is the fourth most common cancer in women, with an estimated 528,000 new cases and 275, 000 deaths in year 2012 (3). Incidence of cervical cancer peaks at around age 65 years in the U.S. among women with an intact uterus (2). Cervical cancer accounts for 7.5% of all female cancer deaths globally (3). The majority (> 80%) of the global burden occurs in the less developed regions such as in Africa, South Asia, Central and Soutern America where proper routine screening is limited or lacking. Rates are lowest in Australia/New Zealand and Western Asia (3).

HPV infection

HPV infection is the most common viral sexually-transmitted infection in the world with a lifetime cumulative risk of >80% (2). Persistent infection of high-risk human papillomavirus (HPV) genotypes is a necessary but not sufficient cause of cervical cancer (4, 5). HPV is reported to be detected in 99.7 percent of cervical cancers (6), including almost all the histologic types such as squamous cell carcinoma (69% of cervical cancers), adenocarcinoma (25% of cervical cancers) and adenosquamous carcinoma (7).In many developed countries, the incidence of squamous cell carcinoma of the cervix has been observed to decrease while that of adenocarcinoma of the cervix is show to be on the rise (8, 9)

NATURAL HISTORY AND EPIDEMIOLOGY OF HPV INFECTION

The virus

HPV belongs to the Papillomavirus genus of Papillomaviridae family. It infects only humans. Currently over 130 HPV genotypes have been identified, with ~ 40 genotypes causing mucosal infections based on tissue tropism (10, 11). The viruses are highly epitheliotropic as productive infections occur only within stratified epithelia of skin, anogenital tract, and oral cavity (10).

HPV is a 8kb, double-stranded DNA virus that has a non-enveloped icosahedral capsid. The HPV genome is circular and contains 8 open reading frames consisting of 6 early (*E1, E2, E4, E5, E6, E7*) and 2 late (*L1, L2*) genes. The *E6, E7* genes constitute the primary oncogenes. The E6 proteins have been shown to inhibit the transcriptional activity of p53 and the abrogation of p53-induced apoptosis, while the E7 proteins lead to the loss of tumor suppressor (p105Rb) control over E2F transcription factors, resulting in unscheduled DNA synthesis and cellular proliferation (10).

HPV infections of the genital tract have been subclassified into those caused by low-risk HPV genotypes and those caused by high-risk HPV genotypes. At least 13 high-risk/oncogenic HPV genotypes have been identified according to WHO: HPV genotypes16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 (10). Other studies have proposed the following HPV genotypes are high-risk: HPV types 16, 18, 31, 33, 35, 39,

45, 51, 52, 56, 58, 59, 66, 68, 73, and 82 (2, 12). Common low-risk HPV genotypes involved in mostly benign clinical disease includes HPV types: 6 and 11 (most common), 40, 42, 43, 44, 53, 54, 61, 72, 73, and 81.

HPV life cycle in the female genital tract

Genital HPV infection is transmitted via sexual contact. The viral life cycle is similar in the cervix, vagina and vulva. HPV is believed to access basal epithelial cells at sites of micro-abrasions or injury. Basal cells normally undergo cell division, and the daughter cells migrate from the basement membrane into the suprabasal compartment. Under normal conditions, these cells initiate terminal differentiation as they migrate to the superficial epithelial layers. When basal cells are infected by HPV, early HPV genes *E1*, *E2, E4, E5, E6*, and *E7* are expressed, and viral DNA replicates from the circular viral genome which normally resides as a nuclear plasmid in the cells. The infected suprabasal cells fail to withdraw from the cell cycle and continue to support DNA synthesis. Late genes *L1*, *L2*, are expressed in these infected cells as they migrate into the upper epithelial layers, where progeny virions are formed in the nuclei. These viruses are then shed from the epithelium and can initiate new infection (10, 13).

Most HPV infections spontaneously resolve without clinical disease within 8-12 months, and viral load is usually reduced to undetectable levels by 2 years after infection (2, 14).

Progression of HPV infection to pre-malignant and malignant lesions

HPV infection has been implicated in benign and malignant lesions in the genital tract. Low-risk HPV types 6 and 11 are commonly found in benign warts (condylomata acuminata). Persistence of high risk HPV infection at 1-2 years after initial infection has been shown to be highly predictive of a lifetime risk of pre-invasive and invasive cervical neoplasia (15). The most common HPV types involved in cervical cancer are HPV types 16 and 18 (10).

Figure 2.1 (Figure 2.1) is a schematic diagram illustrating the progression of persistent high-risk HPV infection to pre-malignant and malignant cervical lesions. The major steps include persistence of HPV infection, progression to low-grade intraepithelial lesions (LSILs), high-grade intraepithelial lesions (HSILs), carcinoma in situ and eventually invasive cancer. LSILs reflect productive viral replication. Less than 15% of LSIL progresses to HSIL and about 10-30% of HSIL eventually progresses to invasive cancer if left untreated (16-18). The progression of these untreated lesions to microinvasive and invasive cancer is associated with two events: (i) Inhibition of the differentiation process which leads to a cellular state that cannot support the normal full viral life cycle, (ii) and often integration of the episomal HPV genome into the host chromosomes. The consequences are the loss or disruption of *E2*, and subsequent upregulation of *E6* and *E7* oncogene expression, which confers a growth advantage to these cells with impaired replication control (10).

Factors driving the viral integration are still not fully understood. Recent research suggested that the risk of developing cervical cancer is strongly linked to a particularly vulnerable cell population within squamocolumnar junction (SCJ) of the cervix that have unique morphology and gene-expression profile (19-21). Study showed that expression of junction-specific genes was shared between these SCJ cells and most CIN3 and invasive cancers (20). This expression profile did not appear to be induced by viral oncoproteins; was not regenerated after excision; nor was it detected at HPV-infected sites that did not harbor these SCJ cells such as the vagina or vulva (19-21). The findings suggested that these cells were specific to the cervix and gave rise to the malignant cervical lesions (19-21).

Estradiol has also been shown to stimulate the transcription of HPV type 16 *E6* and *E7* oncogenes in cell lines with integrated HPV 16 (22, 23). Studies have also reported a synergistic mechanism between long-term estrogen exposure and HPV 16 oncogenes that modulates squamous carcinogenesis in the female reproductive tract of transgenic HPV16-expressing mice (24).

Epidemiologic risk factors of cervical cancer and HPV infection are discussed in the next sections.

Epidemiology of HPV infection: Overview

Prevalence of HPV infection is known to peak in the first few years following sexual debut and then decline (4, 25, 26). Concurrent infection by different HPV types is

common (27, 28). In the U.S., the proportion of high-risk versus low-risk HPV types had remained fairly unchanged across different age groups according to the 2003-2006 NHANES survey (29).

A second increase in prevalence has been noted in meta-analyses conducted among older women (over the age of 35 years) in several populations in Africa, Central and Southern America (26, 30). The same trend was observed also in Northern America by de Sanjose et al but not by Bruni et al when more recent studies were included (26, 30). The cause for this second peak in prevalence is not well understood. Several mechanisms have been proposed to explain the prevalence increase, including population-specific cohort effects, re-exposure through new sexual contacts, re-emergence of latent HPV infections at older age, or physiologic changes at the cervix with aging that increase the efficiency of HPV detectability by current sampling methods (25-26, 31-32).

While mid-life changes in sexual behavior has been suggested to be a potential underlying mechanism, recent evidence suggests that it cannot be the sole cause. In a study examining the incident HPV infection among women aged 16-23 years, a nontrivial proportion of women (0-16%) exhibited infection reappearance across HPV types within 3 years of non-detection. Given that over 80% women in that study reported no new sex partners within 6-month- as well as 12-month periods before the date of detection, the reappearance of HPV detection was mostly attributed to changes of HPV detectability in the cervical tract, which in turn might be influenced by factors other than sexual behavioral characteristics and HPV acquisition, such as fluctuations in

immunocompetence levels (33). Another study conducted among HIV-positive women with CD4 cell count <200 cells/mm^3 found a high rate of incident HPV detection in those who were sexually inactive for at least 18 months (22%) , which was not substantially lower than that of women who were sexually active (34). These data reflect potential reactivation of latent HPV infection that can become detectable in the setting of immunosuppression.

While sexual behavioral risk factors is believed to be the main driving factor of acquisition of new HPV infection, factors which predispose to HPV persistence and/or carcinogenesis are not completely known. Host factors such as immune suppression (e.g. HIV), menopause, infection by other sexually transmitted infection such as chlamydia, and viral factors such as coinfection with other HPV types and high viral load have been noted to be associated with persistent HPV infection (4, 5, 13, 35). Environmental risk factors which have been found to be associated with persistent HPV infection include cigarette smoking, high parity, and oral contraceptive use (4, 5, 36).

The rest of this section will focus on current findings on the association of hormonal contraceptive use with HPV infection and cervical cancer.

Hormonal contraceptive use as a risk factor for HPV infection and cervical cancer

Association of oral contraceptive (OC) use and HPV infection

There is a general consensus on the risk of long-term combined oral contraceptive (COC) use on invasive cervical cancer or high-grade cervical neoplasia (37-42). According to a pooled analysis performed by the International Collaboration of Epidemiological Studies of Cervical Cancer (IARC) that comprised over 1500 HPV-positive cervical cancer cases and 200 controls, relative to never users of COCs, 5 or more years of COC use is associated with a significant 2-fold increased risk of invasive cervical cancer after adjustment for other risk factors (37). The risk declines to that of never users after cessation of use for more than 10 years (37). Similar association was observed in a pooled analysis of additional case–control and cross-sectional studies (40, 41).

It remains unclear whether the association between COC and cervical cancer is driven by COC's effect(s) on carcinogenesis and/or on upstream events such as HPV acquisition and persistence.

Long-term OC use has been suggested in studies to increase the risk of both prevalent and persistent HPV infections. For instance, a recent study done in women aged 20-37 years in Thailand (n=1250) showed that, relative to never users, long-term and recent use of COC was associated with a statistically significant 1.9-fold increase in overall adjusted HPV baseline prevalence, and a statistically significant 36%-reduction in HPV clearance after adjustment for cytological diagnosis and other risk factors for HPV infection (43, 44).

Conclusions, however, have been inconsistent across cohort studies. Current findings on the association between OC/COC use and HPV acquisition, persistence and prevalence are summarized in tables 2.1-2.2 (Tables 2.1-2.2). Although some studies demonstrated a positive association between OC use and HPV persistence (44, 45), negative or null associations were observed in other studies (46-52). A few studies reported that OC use was associated with increased HPV acquisition (53, 54), but the findings were not replicated in other studies (44, 47, 48, 55-60). Inconsistency of findings has also been seen across studies looking at the association between OC use and HPV prevalence. A recent systematic review and a pooled analysis noted that neither a strong positive or negative association could be concluded between OC use and HPV prevalence (61, 62). Mixed findings with positive association (31, 43, 63-68) or null association (58, 69-86) have been reported in other studies.

Association between HPV infection and use of other hormonal contraceptives

There have been relatively few reports made on the association between use of other hormonal contraceptives and HPV infection or cervical cancer. According to a pooled analysis performed by IARC, the relative risk of invasive cervical cancer is moderately raised at 1.2-fold in long-term users versus never-users of progestagen-only injectable contraceptives (37).

How the use of progesterone derivatives may influence the natural history of HPV is not well defined as related data are limited. In a large cross-sectional study conducted among women residing at the U.S.-Mexico border (n=2246), investigators found that ever use of

Norplant and current use of injectable contraceptives were respectively associated with 2.4-fold increased odds (95% CI: 0.9-6.0) and 2.2-fold increased odds (95% CI: 1.4-3.6) of oncogenic HPV prevalence after adjusting for sexual behavior (87). Two other cross-sectional studies performed in the U.S. also reported positive associations of HPV prevalence with DMPA (63, 74).

A recent 1-year prospective cohort study conducted among women with low-grade or equivocal cytological abnormalities in the U.S. (n= 2408) showed a marginal1.2-fold increase in odds (95% CI: 1.0-1.3) of persistent HPV infection among current users versus never users of injectable hormonal contraceptives (49), suggesting that use of progesterone derivatives may potentially increase the risk of cervical cancer by promoting HPV persistence.

Findings, however, are mixed across studies, as summarized in tables 2.3 and 2.4 (Tables 2.3, 2.4). No association was observed between HPV prevalence and intrauterine-device in a pooled analysis including 10 case-control studies and 16 prevalence studies (88). Multiple other studies also showed no significant associations with HPV prevalence (43, 69, 70, 75, 71, 86, 89), and with HPV incidence or persistence (44, 46, 49, 50, 54).

Heterogeneity in reported associations between HPV infection and hormonal contraceptive use across epidemiologic studies

Heterogeneity in study findings are likely related to differences in study populations, study designs, as well as different characterizations of hormonal contraceptive use in data

collection and analyses. Residual confounding of sexual exposure to HPV, which might exist to different extent in studies due to variations in designs and population characteristics, can also contribute to the heterogeneity of reported data.

The inconsistencies may also be partly explained by the intrinsic limitation of most HPV natural history studies in defining a persistent infection and an incident infection. Testing intervals in studies were seen to range from 2 months to 7 years, with a median of around 6 months (13). Since specific HPV status of study participants prior to study entry is often not available for assessment, the distinction between a persistent and transient infection is arbitrary as it depends on the sampling frequencies and the time points of sampling in relation to the natural history of infection. Defining a true incident infection can also be challenging as an "incident" detection of HPV can represent a newly acquired HPV subtype, a recurrent infection of an HPV subtype that has cleared before study entry, or a reactivation of a "latent" infection detected at prior time points in the study. This limitation will be further discussed in Chapter 6 (Figure 6.2).

Moreover, published data on the association between hormonal contraceptive use and HPV infection have mostly been focused on the use of OCs among women below the age of 40 years, or across a broad age spectrum with relatively small proportion of the study population in the older age group (Tables 2.1-2.4).

It is therefore difficult to draw definite conclusions on the independent role(s) of the different types of hormonal contraceptives in the natural history of HPV based on current

data, particularly among women aged 40 years or above who have not been the focus in most published studies, and whose sexual behavioral profiles are different from those of younger women (90).

HPV infection and host immune response

HPV is known to evade immune detection without inducing proinflammatory response in the keratinocytes via a number of mechanisms. Viral replication stays intraepithelial with little or no release into the local milieu of proinflammatory cytokines. There is no viremic or blood-borne phase in its life cycle. Free virus particles are shed from the surface of squamous epithelia with poor access to blood or lymph nodes where immune responses are initiated. Expression of the HPV oncogenes *E6* and *E7* has been shown *in vitro* to suppress interferon response, resulting in downregulation of chemokines and decreased recruitment of Langerhan cells (10, 91). HPV 16 E6 and E7 proteins have also been observed to downregulate *in vitro* expression of toll-like receptor 9, which mediates innate immune responses against bacterial and viral pathogens (92).

Nevertheless, a primary HPV-infection is usually cleared naturally in approximately 90% of cases (93). Both humoral and cell-mediated responses are elicited in the host against HPV infection. Antibody responses to HPV infection are type-specific. The host's humoral IgG immune response to HPV infection is usually weak and varied among women. More than 40% of women do not seroconvert or the antibodies wane over time, suggesting that antibodies elicited by natural infection may not provide complete protection to HPV over time (93, 94).

Cell-mediated immune response has been implicated in the clearance of HPV infection (95, 96, 97), but its exact mechanisms have not be fully elucidated. Studies have evaluated immune markers in cervical secretions (98-103), tissue-based immune markers (104-108), immune responses in patient-derived peripheral blood mononuclear cells (PBMCs) (109-113), and circulating immune markers in plasma or serum (114-119) to characterize immune responses against HPV infection. Main findings of these selected studies are summarized in table 2.5 (Table 2.5).

Findings of these immune marker measurements have been largely inconsistent. This can be partly explained by the lack of consistency in sample collection and cytokine measurement methods, as well as outcome definitions used for analyses. The discrepancies could also stem from differences in the study populations, e.g. young versus old women. Furthermore, many studies focused on only one or few cytokines or immune marker, making it challenging to explore the interactions of all immune players involved.

FEMALE SEX HORMONES AND HOST IMMUNE RESPONSES

Association between HPV detection and endogenous female sex hormonal profile

Persistence of HPV infection was reported to associate with old age in postmenopausal women (35). Lymphoproliferative responses in women older than 45 years was found to be poorer among those with persistent HPV infection, suggesting that inadequate

immunologic control of HPV infection may result in impaired clearance of HPV as women age through menopause (119).

Female sex hormones include estrogen and progesterone. In females, estrogen and progesterone fluctuates due to variation in concentrations of pituitary luteinizing hormone (LH) and follicle stimulating hormone (FSH) during menstrual cycle. During follicular phase of the menstrual cycle, increase in 17 beta-estradiol and decrease in progesterone plasma concentrations are seen, while high plasma 17 beta-estradiol and progesterone concentrations occur in the luteal phase (120).

Among premenopausal women, study findings have suggested a menstrual cycle effect on HPV detection based on intrapersonal variability in HPV detection observed in non-pregnant women (121-125). However, the evidence has been inconsistent. A few studies reported the follicular phase being associated with a peak in HPV detection (123-125), whiles others observed no association (125-127), or reported increased detection at midcycle (128), during luteal phase (122), or in the periovulatory phase (121).

Several hypotheses have been proposed to explain the possible association between the menstrual cycle and HPV detection. Estrogen may facilitate thickening and maturation of cervical epithelium resulting in more shedding of virus. Bacterial flora tends to be more stable at midcycle, which may contribute to the temporal variability observed with HPV detection during menstrual cycle (129). Suppression of local mucosal immunity

due to anti-inflammatory effects of sex hormones has also been thought to increase risk of HPV persistence (128, 130-135).

Role of estrogen and progesterone in immune regulation

Both estrogen and progesterone receptors are present on cervical epithelial cells. Exposure to these hormones has been suggested to influence the natural history of HPV infection in experimental studies.

Progesterone was shown to affect immune responses by (i) skewing cytokine production towards a general Th2 response (136), (ii) inhibiting cytotoxic T cell activity, (iii) impairing NK T cell activity (137-139), (iv) reducing NK cell function and Fcγ R expression on monocytes and thereby impairing antibody-dependent cell cytotoxicity (139-141), and (iv) possibly affecting innate response by inhibiting TLR9-induced IFNγ production by human and mouse plasmacytoid dendritic cells (142). Mice treated with DMPA exhibited decreased levels of HSV-2 specific mucosal immune responses after intravaginal immunization with TK-HSV2 (143). Rhesus macaques treated with DMPA suffered from increased SIV acquisition by more than 7-fold and had significantly increased viral shedding during the acute phase of infection (144).

The effect of estrogen can either be proinflammatory or anti-inflammatory depending on its concentrations (145). At low concentrations ($<10^{-8}$M), estrogen has the ability to induce TNFα, IL-6 and IL-1β expression, and increases migration of leukocytes to the

site of inflammation (139, 145). At high concentrations (>10^6M), estrogen was shown to inhibit cellular immunity by impairing cytotoxic T cell activity(146) and decreasing migration of T cells and macrophages into the genital tract by downregulating expression of adhesion molecules including ICAM-1, VCAM-1 and E-selectins (139, 145).

During the menstrual cycle, fluctuations in immune marker expression have been observed. In late follicular and ovulatory phase when estrogen level is highest, PHA-L stimulated PBMCs and cervical mucus from women were noted to have increased IL-10 and decreased IL-1β expression (147). Populations of FoxP3+CD4+CD25+ T cells were also noted to increase in peripheral blood mononuclear cells (PBMCs) from women in late follicular and ovulatory phase (148). In the luteal phase of menstrual cycle when progesterone concentration increases, suppression of proinflammatory cytokines including IL-6, TNF-α and reduced cytotoxic activity have been observed (149).

Hormonal contraceptive use and host cytokine profiles

Use of hormonal contraceptives appears to influence local cytokine profiles in cervical tract based on data from population studies. Current users of OCs were found to have increased expression of INF-γ, IL-12 as well as IL-10 in cervical secretion after controlling for sexual behavioral characteristics and other risk factors for HPV (150, 151). Another study of COC users showed increased IL-10, IL-4, IL-6 but decreased concentrations of IL-2, IL-12 and INF-γ (152). Such inconsistency in findings is commonly seen among cytokine studies (91). The discrepancies are likely due to

variation in study designs, different sampling and testing methodologies, and interpersonal pharmacokinetic differences.

A general pattern of suppressed proinflammatory response associated with female sex hormones has been reported in experimental studies. For instance, PBMCs from COC users were found to have lower concentrations of TNF-α (134), IFN-γ (153) and NK T cell activity (141). Treatment of PBMCs *in vitro* with biologically relevant concentrations of estrogen and progesterone was noted to lower expression of proinflammatory cytokines and increase expression of anti-inflammatory cytokines after exposure to HPV16 virus-like-particles (135).

Current findings on cervical cytokines in relation to hormonal contraceptives are, however, limited in at least two ways: Most of the published findings were based on cervical samples collected from adolescents or young women. It is unclear how these findings would translate to HPV detection in older women. In addition, cervical cytokine evaluation in these population studies has mostly been restricted to a few cytokines, which might not fully capture the activities and complex interactions of all the major cervical cytokines involved in the defense against HPV infection. Exploratory data on the levels of these other cervical cytokines in relation to HPV infection and hormonal contraceptive use are lacking. Further assessment in this regard is needed to facilitate more intensive evaluation of cervical cytokine dynamics in the setting of HPV infection.

HPV TESTING IN CERVICAL CANCER SCREENING

The incidence of cervical cancer is about 5-fold lower in the developed nations, e.g. U.S. and western Europe, relative to developing countries due to the implementation of routine cervical cancer screening in the population.

Figure 2.5 summarizes the current recommendations for cervical screening with respect to different age groups in the U.S. The role of HPV testing in cervical screening has expanded since its first introduction in the 1990s. At present, co-testing with HPV and Pap smear every 5 years (preferred) or Pap testing alone every 3 years (acceptable) is recommended as the standard of care for women aged 30 to 65 years based on screening guidelines from the American Cancer Society, American Society of Colposcopy and Cervical Pathology, American Congress of Obstetricians and Gynecologists, and U.S. Preventive Services Task Force (154, 155) (Figures 2.2A, B).

Recently in April 2014, the U.S. Food and Drug Administration approved the Cobas HPV test (Roche Diagnostics) as a primary screening tool for cervical cancer for women aged 25 years or older, further expanding the role of HPV testing in cervical cancer prevention. While no formal guidelines have been issued from professional societies on the appropriate testing intervals for primary HPV testing, interim recommendations have been proposed by experts to guide clinical practice (155, 156) (Figure 2.2C). Current data indicate that HPV testing alone is at least equal to Pap testing alone at 3-year intervals in women aged 30 years or older (155). However, primary HPV testing in

women aged 25 to 29 years would have higher false positive rates as many young women have transient HPV infections that do not cause cervical dysplasia.

Among older women aged over 35 years, HPV testing is part of the routine screening regimen for cervical cancer. A positive HPV test can be challenging to interpret in this age group due to a number of reasons. Study has shown a loss of correlation between HR-HPV test results and cytologic abnormalities as age increases, likely due to increased proportion of ASCUS and reduced prevalence of HR-HPV detected in older women (157). In addition, current understanding is limited in identifying (i) determinants of persistent high-risk HPV infection, (ii) biologic roles that these risk factors may play in the host; and (iii) whether these HPV detections represent just merely transient HPV detections with little potential to progress to pre-malignant or malignant lesions.

Further natural history and laboratory studies are therefore warranted among older women (aged >35 years) to better understand the risk factors driving prevalent HPV detection in this age group.

CONCLUSIONS

A brief literature review has been provided in previous sections on the natural history of HPV infection, its association with cervical cancer and its association with hormonal contraceptive use. The main findings and knowledge gaps are summarized in table 2.6 (Table 2.6).

Given these findings, a conceptual model showing the potential associations among hormonal contraceptive use, HPV detection, and the cervical immune milieu in women older than the age of 35 years is outlined in figure 2.3 (Figure 2.3).

Based on this conceptual model, we proposed the following specific aims and hypotheses for this dissertation research conducted in pre- and perimenopausal women aged above 35 years in the U.S.:

Aim 1: To determine the association of hormonal contraceptive use with the prevalence of HPV DNA detection

Hypothesis: Longer and more recent use of hormonal contraceptives is associated with higher prevalence of HPV DNA detection.

Aim 2: To estimate the association between hormonal contraceptive use and incident HPV DNA detection

Hypothesis: Use of hormonal contraceptives is associated with greater odds of incident HPV detection.

Aim 3: To describe and compare differences in the host cervical cytokine profiles between current hormonal contraceptive users and non-current users

Hypothesis: The cervical cytokine profile in current hormonal contraceptive users is disrupted and different from that seen in non-current contraceptive users.

Methods and results are shown and discussed in Chapters 3, 4, 5.

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TABLES AND FIGURES

Table 2.1. Summary of prospective study findings on the association of oral contraceptive (OC) use with HPV infection

									Association with HPV after controlling for confounding factors		
Study	**Study design**	**Study area(s)**	**Study population**	**Age range**	**Exposure of interest**	**OC duration assessed?**	**OC recency assessed?**	**Control for confounding by other factors?**	**Prevalence**	**Acquisition of new HPV type**	**Persistence**
Nielsen et al 2009 (54)	Prospective	Denmark	Population-based (n=5448)	20-29	OC	Yes	Yes	Yes	na	Positive with current use and >6 years of use	na
Winer et al 2003 (53)	Prospective	U.S.	Female university students (n=603)	18-20 Mean: 19.2	OC	No	Yes	Yes	na	Positive with current use	na
Ho et al 2002 (55)	Prospective	U.S.	Female university students who has history of HPV-DNA-positivity (n=608)	Mean: 20 (SD: 3)	OC	No	No	Yes	na	Negative	na
Moscicki et al 2001 (56)	Prospective	U.S.	Women attending family planning clinics (n=601)	13-21	OC	No	Yes	Yes	na	Negative	na
Syrjanen et al 2006 (58)	Prospective	Former Soviet Union	Women presenting at gynecologic or STD clinics (n=3187)	15-85 Mean: 32.6	OC	No	No	Yes	na	Null	na
Sycuro et al 2008 (59)	Prospective	U.S.	Female university students (n=147)	Mean: 20.5 (SD:1.3)	COC	No	Yes	Yes	na	Null	na
Shew et al 2006 (60)	Prospective	U.S.	Females attending adolescent health clinics	14-17 Mean: 15.3	COC	No	Yes	Yes	na	Null	na
Sellors et al 2003 (57)	Prospective	Canada	Women presenting for routine screening (n=307)	15-49 Mean:33	COC	No	Yes	Yes	na	Null	na
Marks et al 2011 (44)	Prospective	Thailand	Women with normal cytology (n=1135)	20-37 Mean: 29.6	COC	Yes	Yes	Yes	na	Null	Positive
Vendenvelde et al 1992	Prospective	Belgium	Women with normal cytology (n=323)	No details given	OC	No	No	Yes	na	na	Positive for HPV types

(45)											16,18, 33
Molano et al 2003 (46)	Prospective	Columbia	Women with normal cytology (n= 1995)	18-85 Median: 29	OC	No	No	Yes	na	na	Negative
Schmeink et al 2010 (47)	Prospective	The Netherlands	Population-based sample (n=1812)	Mean: 23.2 (3.3)	OC	No	No	No	Null	Null	Null
Goodman et al 2008 (48)	Prospective	U.S.	Women attending gynecologic clinics (n=972)	18-85 Mean: 35	OC	Yes	Yes	Yes	na	Null	Null
Maucort-Boulch et al 2010 (49)	Prospective	U.S.	Women with equivocal, ASCUS or LSIL (n=2019)	18-≥50 Mean: 24.9	OC	No	No	Yes	na	na	Null
Muñoz et al 2009 (50)	Prospective	Colombia	Women with normal cytology (n=1728)	18-85	OC	No	No	Yes	na	na	Null
Lai et al 2008 (51)	Prospective	Taiwan	Women with normal cytology (n=412)	29-75 Median: 47	OC	No	Yes	Yes	na	na	Null
Richardson et al 2005 (52)	Prospective	Canada	Female students attending university health clinics (n=621)	17-27+ >80% less than 26	OC	Yes	No	Yes	na	na	Null
na: not assessed; OC: oral contraceptives; COC: combined oral contraceptives											

Table 2.2. Summary of cross-sectional and case-control study findings on the association of oral contraceptive (OC) use with HPV prevalence

									Association with HPV after controlling for confounding factors
Study	**Study design**	**Study area(s)**	**Study population**	**Age range**	**Exposure of interest**	**OC duration assessed?**	**OC recency assessed?**	**Control for confounding by other factors***	**HPV Prevalence**
Green et al 2003 (61)	Systematic review of 19 studies	12 countries in Europe, Asia North,Central and South America	N=20 509	18- 50+	OC	Yes	Yes	Yes	Null
Vaccarella et al 2006 (62)	Pooled analyses of IARC HPV prevalence surveys	14 countries in Asia, Central America, South America, Europe	N=15,145	15- 65+ Mean: 40.9 yrs	OC	Yes	Yes	Yes	Null
Ghanem et al 2011 (63)	Cross-sectional	U.S.	Women for routine Pap screening at primary care, STD and family planning clinics (n=7718)	14-65 >80% between 18-45	OC	No	Yes	Yes	Positive with current OC use
Herrero et al 2005 (31)	Cross-sectional	Costa Rica	Population-based study (n=8513)	<25 to 65+ with no specific range given, >67% below the age of 44	OC	No	Yes	Yes	Positive with current OC use
Molano et al 2002 (64)	Cross-sectional	Colombia	Population-based sample (n=1859)	18-85 Median: 32	OC	No	Yes	Yes	Positive with current OC use
Marks et al 2011 (43)	Cross-sectional	Thailand	Women with normal cytology (n=1070)	20-37 Mean: 29.6	COC	Yes	Yes	Yes	Positive with current and ≥6 years of use (with both oncogenic and any HPV)
Rousseau et al 2000 (65)	Case-control study	Brazil	Women participating in a maternal child care program (n=1425)	18-60 Mean: 33.3	OC	Yes	No	Yes	Positive with ≥ 6 yeas of use
Ley et al 1991 (66)	Cross-sectional	U.S.	University females presenting for routine pap smears (n=467)	17-50 Mean: 22.9	OC	Yes	No	Yes	Positive with more than 1 year of use

Kjaer et al 1997 (67)	Cross-sectional	Denmark	Population-based sample with normal cytology (n=956)	20-29 Median: 25	OC	Yes	No	Yes	Positive with ≥5 years of use
Nielsen et al 2008 (68)	Cross-sectional	Denmark	(i) Age 40-50 years: n=1443 (ii) Age 20-29 years: n=10544	(i) 40-50 (ii) 20-29 Mean or median not given.	OC	No	Yes	Yes	(i) 40-50 years: Null (ii) 20-29: Positive
Kasap et al 2011 (69)	Cross-sectional	Turkey	Women presenting for pap smears at clinics (n=642)	15-65 Mean: 36	OC	No	No	Yes	Null
Li et al 2010 (71)	Cross-sectional	China	Population-based sample in Beijing (n=6385)	25-54 Mean: 39.6	OC	No	No	No	Null
Garvric et al 2010 (70)	Cross-sectional	Solvenia	Women with CIN at baseline (n=1435)	No range given Mean: 35.7 (SD 9.8)	OC	No	No	No	Null (only assessed HPV 16 and 18)
Ripabelli et al 2010 (72)	Cross-sectional	Italy	Women present for routine pap smears (n=299)	18-63 Median: 34	OC	No	No	No	Null
Smith et al 2010 (73)	Cross-sectional	Madagascar	Female sex workers (n=90)	18-58 Mean: 32.6	OC	No	No	Yes	Null
Harris et al 2009 (74)	Case-control	U.S.	Women attending gynecologic clinics (n=257 for analysis between HPV-positive and negative women)	18-50 >70% between 20-29 No mean or median given	COC	Yes	Yes	Yes	Null
Syrjanen et al 2006 (58)	Cross-sectional	Former Soviet Union	Women presenting at gynecologic or STD clinics (i) Age <25 years: n=1103 (ii) Age 26-55 years: n=2004 (iii) Age >55 years: n=80	Means: (i) <25 yrs: 21.7 (ii) 26-55 yrs: 37.3 (iii) >55 yrs: 61.6	OC	No	No	Yes	Null for all groups (only assessed HR-HPV)
Deacon et al 2000 (75)	Case-control	United Kingdom	Women from screening clinics and general practitioners' office (n=583)	Overall range from <20 to 50+; Over 70% in the range of 25-44.	OC	Yes	Yes	No	Null
Peyton et al 2001 (76)	Cross-sectional	U.S.	Women with no history of abnormal pap smear one year prior to baseline (n=3863)	18-40 Mean: 28	OC	No	No	Yes	Null
Hankins et al 1999 (77)	Cross-sectional	Canada	Women with HIV (n=375)	16.5–77.3 Median: 32.5	OC	No	No	No	Null

Veresse et al 1992 (78)	Cross-sectional	Hungary	Women with normal cytology (n=425)	18-58 Mean: 30.1	OC	Yes	No	Yes	Null (only assessed HPV 6, 11, 16, 18)
Burkett et al 1992 (79)	Cross-sectional	U.S.	Women attending Student Health Clinic (n=465)	17-39 Mean:22.7	OC	No	Yes	No	Null
Bauer et al 1993 (80)	Cross-sectional	U.S.	Women with normal cytology	16-81 Median: 34	OC	No	Yes	Yes	Null
Hildesheim et al 1993 (81)	Cross-sectional	U.S.	Women attending gynecologic clinics (n=263	16-72 Median: 26	OC	No	Yes	Yes	Null
Wheeler et al 1993 (82)	Cross-sectional	U.S.	Female students attending university health clinic with normal cytology (n=357)	18-47 Median: 23	OC	No	Yes	Yes	Null
Fairley et al 1994 (83)	Cross-sectional	Australia	Women attending gynecologic clinics (n=298)	18-35 Mean: 27	OC	No	Yes	No	Null
Agorastos et al 1995 (84)	Cross-sectional	Greece	Women with normal cytology (n=226)	20-55 Mean or median not given	OC	No	No	No	Null
Munoz et al 1996 (85)	Cross-sectional	Spain, Colombia, Brazil	Women with normal cytology (n=1184)	Spain: 41.7 Colombia: 42.8 Brazil: 52.7	OC	Yes	No	No	Null
Kataja et al 1993 (86)	Case-control	Finland	Women attending community health care clinics (n=1397)	14-67 Mean: 28.1	OC	No	No	No	Null
na: not assessed; OC: oral contraceptives; COC: combined oral contraceptives									

Table 2.3. Summary of cross-sectional and case-control study findings on the association between progestin-only (POC) contraceptive use and HPV prevalence

Study	Study type	Study area(s)	Study population	Age (years)	Exposure of interest	POC duration assessed?	POC recency assessed?	Control for other risk factors?	Association with HPV prevalence
Castellsagué et al 2011 (88)	Pooled analyses of 10 case-control studies and 16 prevalence surveys	14 countries in Asia, Central America, South America, Europe	2205 women with cervical cancer, 2214 matched control women without cervical cancer, 15 272 healthy women	Mean: 41.3 (HPV+) 37.8 (HPV-)	IUD	Yes	No	Yes	Null
Ghanem et al 2011 (63)	Cross-sectional	U.S.	Women for routine Pap screening at clinics (n=7718)	14-65 >80% between 18-45 No mean or median given	DMPA	No	Yes	Yes	Positive
Harris et al 2009 (74)	Case-control	U.S.	Women attending gynecologic clinics (n=257 for analysis between HPV-positive and negative women)	18-50 >70% between 20-29 No mean or median given	DMPA	Yes	Yes	Yes	Positive (with oncogenic HPV)
Giuliano et al 2002 (87)	Cross-sectional	U.S.	Women attending family planning clinics (n=2246)	15-79	(i) DMPA (ii) Norplant	No	Yes for DMPA	Yes	Positive: DMPA and any and HR-HPV Positive: Norplant and LR-HPV Borderline association: Norplant and HR-HPV
Burkett et al 1992 (79)	Cross-sectional	U.S.	Women attending Student Health Clinic (n=465)	17-39 Mean:22.7	IUD	No	Yes	No	Positive
Agorastos et al 1995 (84)	Cross-sectional	Greece	Women with normal cytology (n=226)	20-55 Mean or median unclear	IUD	No	No	No	Positive
Marks et al 2011 (43)	Cross-sectional	Thailand	Women with normal cytology (n=1070)	20-37 Mean: 29.6	DMPA	Yes	Yes	Yes	Null
Kasap et al 2011 (69)	Cross-sectional	Turkey	Women presenting for pap smears at clinics	15-65 Mean: 36	IUD	No	No	No	Null

			(n=642)						
Garvric et al 2010 (70)	Cross-sectional	Solvenia	Women with CIN at baseline (n=1435)	Mean: 35.7 (SD 9.8)	IUD	No	No	No	Null
Li et al 2010 (71)	Cross-sectional	China	Population-based sample in Beijing (n=6385)	25-54 Mean: 39.6	IUD	No	No	No	Null
Dai et al 2006 (89)	Cross-sectional	China	Population based sample in Shan Xi (n=662)	15-59 60% < age of 44	IUD	No	No	No	Null
Deacon et al 2000 (75)	Case-control	United Kingdom	Women were recruited from screening clinics and general practitioners' office (n=583)	<20-50+. Over 70% in the range of 25-44. Mean, median or specific age range not given.	Progestin-only pills	No	No	No	Null
Kataja et al 1993 (86)	Case-control	Finland	Women attending community health care clinics (n=1397)	14-67 Mean: 28.1	IUD	No	No	Yes	Null

Table 2.4. Summary of prospective study findings on the association between progestin-only contraceptive (POC) use and HPV infection

									Association with HPV after controlling for confounding factors		
Study	**Study type**	**Study area(s)**	**Study population**	**Age (years)**	**Exposure of interest**	**POC duration assessed?**	**POC recency assessed?**	**Control for other risk factors?**	**Prevalence**	**HPV Acquisition**	**HPV Persistence**
Maucort-Boulch et al 2010 (49)	Prospective	U.S.	Women with equivocal, ASCUS or LSIL (n=2408)	18-50+ Mean: 24.9	DMPA	No	No	No	na	na	Positive
Maucort-Boulch et al 2010 (49)	Prospective	U.S.	Women with equivocal, ASCUS or LSIL (n=2408)	18-50+ Mean: 24.9	Norplant	No	No	No	na	na	Null
Marks et al 2011 (44)	Prospective	Thailand	Women attending family planning clinics (n=1135)	20-37 Mean: 29.6	DMPA	Yes	Yes	Yes	Null	Null	Null
Molano et al 2003 (46)	Prospective	Columbia	Women with normal cytology (n= 1995)	18-85 Median: 29	IUD	No	No	No	na	na	Null
Muñoz et al 2009 (50)	Prospective	Colombia	Women with normal cytology (n=1728)	18-85 Mean or median unclear	IUD	No	No	No	na	na	Null
Nielson et al 2010 (54)	Prospective	Denmark	Population-based sample (n=9378)	20-29 Mean or median unclear	IUD	No	Yes	Yes	na	na	Null

na: not assessed; POC: progestin-only contraceptives; DMPA: Depot medroxyprogesterone acetate

Table 2.5. Summary of selected study findings on the association of immune markers with HPV infection

Study (since 2000)	Immune marker(s) assessed	Main findings in association with HPV (compared to HPV-negative women)
Study samples: Cervical secretions		
Marks et al 2011 (98)	IFN-γ, IL-2, IL-12, IL-15, IL-4, IL-5, IL-9, IL-13, IL-10, IL-17, eotaxin, IL-1β, IL-1rα, IL-6, IL-8, IP-10, MCP-1, MIP-1α, MIP-1β, RANTES, TNF-α, IL-7, G-CSF, GM-CSF, basic FGF, VEGF, PDGF- bb	Shift in T-cell associated cytokine correlation from IL-2 to eotaxin with HPV infection Elevated IL-5, IL-9, IL-13, IL-17, eotaxin, GM-CSF, and MIP-1α levels seen with HPV
Guha et al 2009 (100)	IL-1β, IL-6, IL-10, IL-12	IL-6 elevated in HPV+ women
Lieberman et al 2008 (101)	IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12(p40/p70), IL-13, IFN-γ	No significant association
Gravitt et al 2003 (99)	IL-10, IL-12	No significant association
Tijong et al 2001 (102)	IFN-γ, TNF-α, IL-1β, IL-12p40, IL-10	Increased detection of IL-12 in HPV+ women
Crowley-Nowick et al 2000 (103)	IL-2, IL10, IL12	IL-12 elevated in HPV+ women
Study samples: Cervical tissue		
Butsch Kovacic et al 2008 (104)	Counts of lymphocytes, neutrophils, macrophages, plasma cells, eosinophils	Increased cervical inflammation with HR-HPV
Bemudez-Morales et al 2008 (105)	IL-10	Increased IL-10 mRNA levels with HPV positivity
Song et al 2007 (106)	IL-6, IL-10, IFN-γ, TNF-α	Increased IFN-γ with HPV
Ortiz-Sanchez et al 2007 (107)	CD80 and 86, IL-10	Decreased CD86 with HPV
Fernandes et al 2005 (108)	IFN-γ, TNF-α, IL-10	Increased IL-10 and IFN-γ with most HPV types except HPV 18
Study samples: Stimulated patient-derived peripheral blood mononuclear cells		
Kemp 2010 (109)	IL-6, IL-8, TNF-α, MIP-1α	Lower IL-6, IL-8, TNF-α, MIP-1α in HPV+ women
Sharma et al 2007 (110)	IFN-γ, IL-2, IL-4, IL-10	Increased IL-4 and IL-10, decreased IL-2 and IFN-γ with HPV infection
Molling et al 2007 (111)	IL-2, cytotoxic T cell activity, iNKT and Treg counts	Increased Treg in women with persistent HPV infection
Seresini et al 2007 (112)	CD4+ T cells, cytotoxic T cell activity, IL-4, IL-5, IL-10, IFN-γ	Level of IFN-γ released predicts infection persistence Higher number of infiltrating CD4(+) and T-bet(+) T cells correlates with a favorable clinical outcome
Bontkes et al 2000 (113)	IL-2, memory cytotoxic T cell activity (mCTL)	Association of mCTL with persistent HPV 16 infection
Study samples: Serum/peripheral blood		
Baker et al 2011 (114)	PBMCs: MIF, sFas, sICAM-1, Svcam-1, IL-8, TNF-α, leptin, HGF, tPAI-1, resistin, adiponectin	TNF-α, IL-8, sFas, resistin were elevated in women with persistent HPV infection
Abike et al 2011 (115)	Neopterin	Lower neopterin in HPV+ women
Kemp et al 2010 (109)	IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-17, IL-1α, IL-1β, IFN-γ, GM-CSF, TNF-α, MCP-1, MIP-1α, IP-10, RANTES, eotaxin,G-CSF, IL-12,	increase in systemic inflammatory cytokines and weak lymphoproliferative responses in HPV+ women with immune deficit

	IL-15, IL-7	
Hong et al 2010 (117)	IFN-γ, IL-6, IL-10, TNF-α	No significant association with clearance of the high risk HPV infection
Bais et al 2005 (118)	IFN-γ, TNF-α, IL-2, IL-4, IL-10, IL-12, sTNFRI, sTNFRII, leukocyte, neutrophils, monocytes, lymphocytes in peripheral blood	Higher mean plasma IL-2 in women with HR-HPV
Adam et al 2009 (119)	Macrophage colon-stimulating factor (CSF-1)	Higher mean CSF-1 levels in women with HR-HPV

Table 2.6. Association of cervical HPV infection with endogenous hormonal profile and hormonal contraceptive use: main findings and knowledge gaps (Ref: 4-153)

Research areas	Main findings	Gaps in knowledge
Hormonal contraceptive use as a risk factor for cervical cancer	• Association has been found between long-term combined oral contraceptive (COC) use and increased risk of invasive cervical cancer. • Long-term use of progestin-only contraceptives is associated with moderately increased risk of invasive cervical cancer.	• Unclear whether the association between COC and cervical cancer is driven by COC's effect(s) on carcinogenesis and/or on upstream events such as HPV acquisition and persistence.

Hormonal contraceptive use as a risk factor for HPV infection	• Long-term , current/recent use of oral contraceptives (OCs) was shown in different cohort studies to associate with
	  • increased HPV prevalence,
	  • increased HPV incidence,
	  • increased HPV persistence.
	• Conclusions have been inconsistent across studies.
		• Paucity of prospective data among women aged 40 years or above.
		• Limited and heterogeneous findings on the associations between other kinds of hormonal contraceptives and HPV infection.

Female sex hormones, HPV detection and host cytokine profile		
Endogenous hormonal profile and HPV detection	• A menstrual cycle effect on HPV detection has been suggested based on intrapersonal variability of HPV detection during menstrual cycle • Findings have been inconsistent across studies • Specific metrics defining menstrual cycle, e.g. serum levels of sex hormones, were lacking in past studies.	• Most of the studies were small, thus limiting statistical inferences. • Unclear how the findings could be interpreted along with other clinical correlates, e.g. exogenous hormonal use.
Hormonal contraceptive use and host cytokine profiles	• Correlations between recent oral contraceptive use and cervical levels of INF-γ, IL-10 and IL-12 have been reported among young women. • A number of past population studies were limited in using serum cytokine levels as surrogate markers for cervical immunity, while serum and cervical cytokine concentrations are not well correlated.. • A general suppression in pro-inflammatory response due to exogenous progesterone and estrogen (low-dose) treatments has been observed in animal and *in vitro* models.	• Most cytokine studies reported in literature were based on samples from young and not older women. • Most studies focused on only one or a few cytokines; unclear whether hormonal contraceptive use is associated with levels of other cervical cytokines involved in cellular immune responses, and if the association differs by days of menstrual cycle.

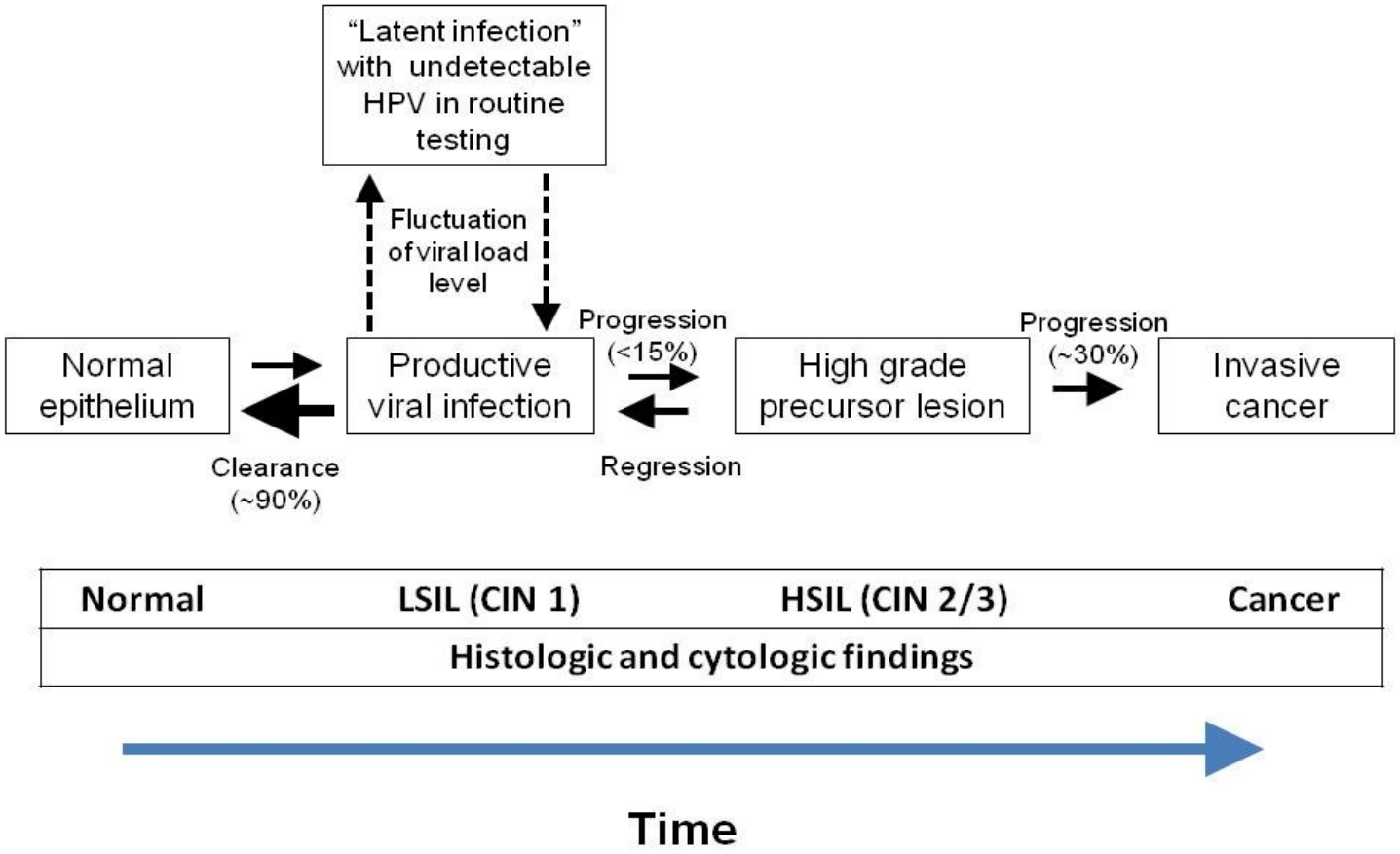

Figure 2.1. Schematic diagram showing progression of HPV infection to pre-malignant and malignant cervical lesions.
CIN: Cervical intraepithelial neoplasia; LSIL: Low grade squamous intraepithelial lesion; HSIL: High grade squamous intraepithelial lesion

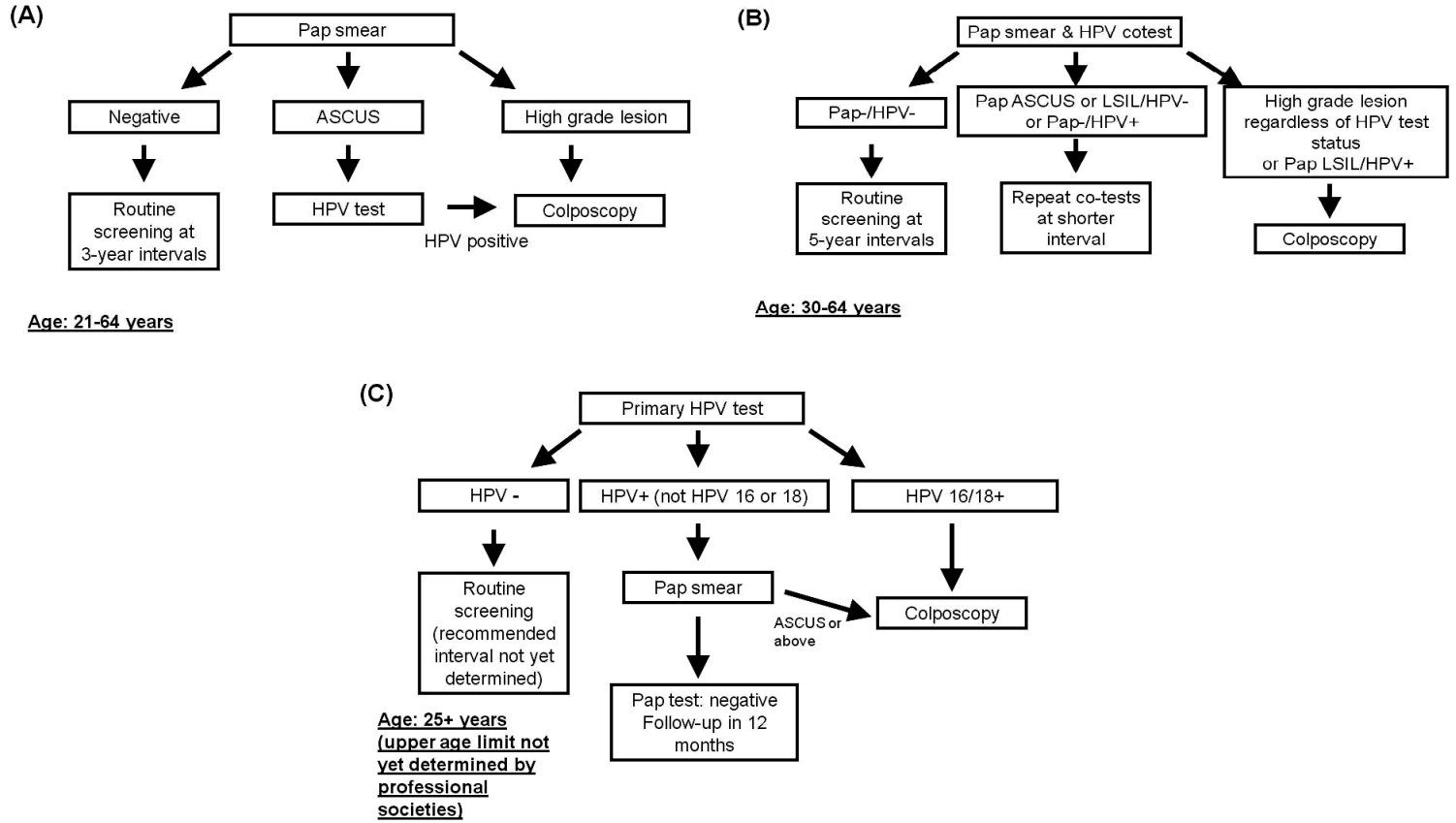

Figure 2.2. A and B: Current cervical cancer screening strategies for women (A) aged 21-64 years and (B) aged 30-64 years according to the recommendations of American Society for Colposcopy and Cervical Pathology (Ref: 154, 155). C. Screening algorithm proposed by experts as interim guidance for primary HPV testing (Ref: 155, 156).

HSIL: High grade squamous intraepithelial lesion. LSIL: Low grade squamous intraepithelial lesion. High-grade lesions: HSIL, atypical glandular cells, atypical squamous cells that favor HSIL, and carcinomas. ASCUS: Atypical squamous cells of undetermined significance.

*** For women with Pap-/HPV+ results, testing for HPV types 16 or 18 with immediate colposcopy for positive results is an alternative.**

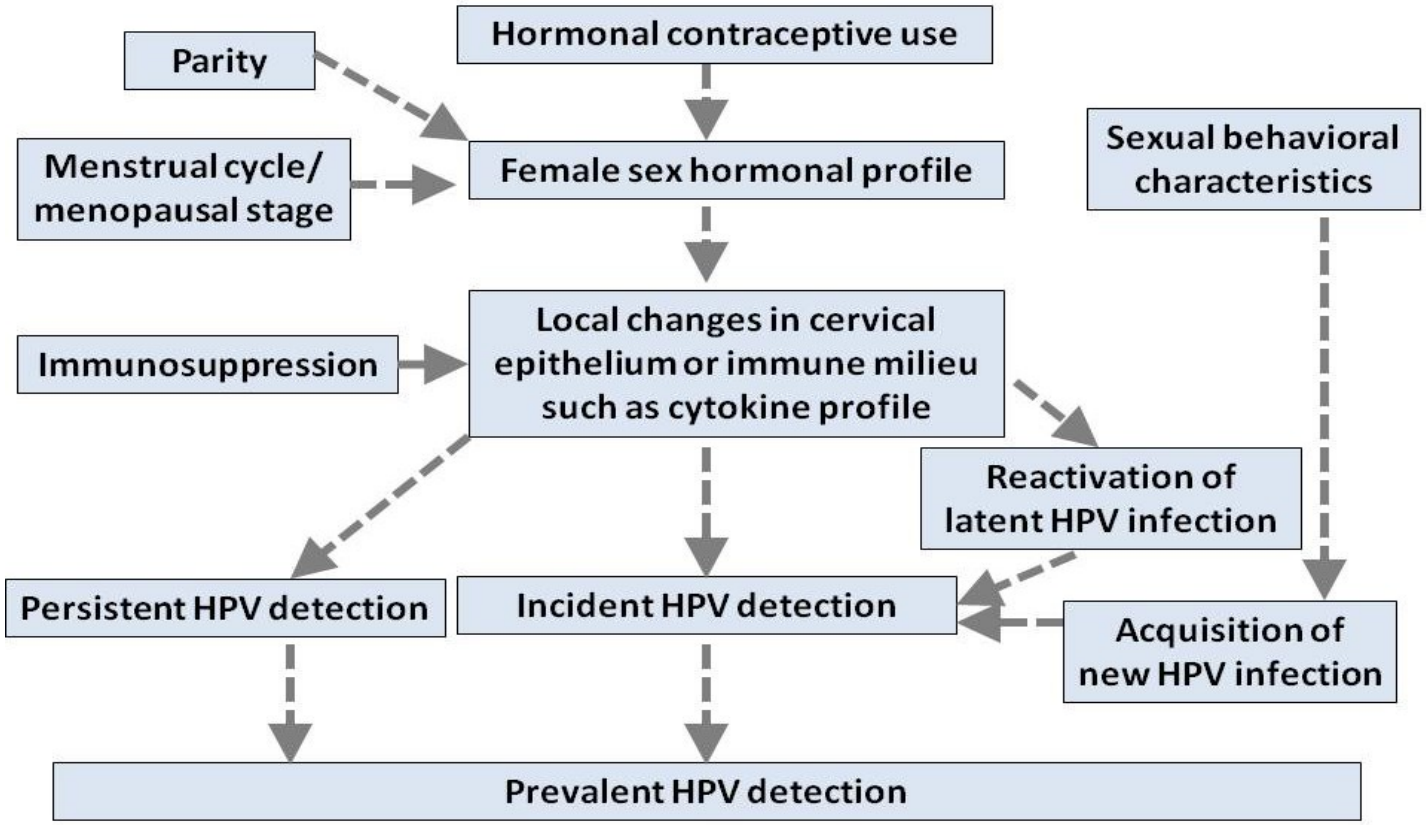

Figure 2.3. Conceptual model showing potential associations among HPV detection, hormonal contraceptive use, and the cervical immune milieu

CHAPTER 3:

Association of Hormonal Contraceptive Use with Cervical HPV Prevalence in Pre- and Perimenopausal Women in the U.S.

by

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ABSTRACT

Background:

Hormonal contraceptive use has been reported to be a risk factor for invasive cervical cancer. Findings have been mixed on its association with HPV infection. Most data were drawn from women aged below 35 years.

Objective:

The aim of this study was to assess the association of hormonal contraceptive use with prevalent cervical HPV detection among pre- and perimenopausal women aged over 35 years who have normal cytology.

Methods:

Data came from a prospective study conducted in women aged 35-60 years in Baltimore MD, USA. Primary exposures of interest were the recency and duration of overall hormonal contraceptive (HC) use, progestin-only contraceptive (POC) use and oral contraceptive (OC) use. Information was collected on HPV genotypes, history of other sexually transmitted infections, Pap smear diagnoses, sexual behavior as well as reproductive characteristics. Prevalence ratios (PRs) with 95% confidence intervals (CIs) were estimated using generalized linear models with Poisson regression.

Results:

A total of 530 peri-menopausal and pre-menopausal women (aged 35-54 years) were included in analyses. Baseline prevalence of any HPV and high-risk (HR)-HPV was 16.4% and 6.4% respectively. Duration or recency of overall HC use did not yield statistically significant association with any HPV or HR-HPV prevalence.

Relative to never POC users, more than 5 years' use of POCs was associated with 3-fold increased prevalence of any HPV after adjusting for days of menstrual cycle, number of lifetime sex partners, acquisition of new sex partner in last 6 months, marital status, time since last abnormal pap smear and history of other sexually transmitted infections in the last 6 months. [adjusted prevalence ratio (aPR): 3.16 (95% CI: 1.82-5.48)]. Association with HR-HPV was also statistically significant with long-term (>5 years) POC use [aPR: 4.26 (95% CI: 1.60-11.30)]. Current POC use was positively associated with HR-HPV (aPR: 2.44 (0.99-5.99) and any HPV (aPR: 1.58 (0.94-2.65) with the estimates bordering on statistical significance.

No significant association was seen between prevalent HPV infection and the recency or duration of OC use in this study population.

Conclusions:

Our findings suggest that long-term use of POCs is associated with a statistically significant increased risk of prevalent HPV detection independent of sexual behavior among women with normal cytology. POC use may have impact on the natural history of HPV infection before development of clinical cervical disease. Longitudinal studies are needed to better define the association and its clinical significance.

INTRODUCTION

HPV infection is one of the most common sexually transmitted infections in the world (1, 2). Persistent infections of oncogenic HPV genotypes are believed to be a necessary but not sufficient cause of cervical cancer (1, 2). Factors which predispose HPV infection to clear or to persist are not completely understood.

Long-term combined oral contraceptive (COC) use has been shown to associate with increased risk of invasive cervical cancer or high-grade cervical neoplasia (3-6). A meta-analysis revealed that, relative to never users of COCs, 5 or more years of COC use is associated with a significant 2-fold increased risk of invasive cervical cancer after adjustment for other risk factors (3). More than 5 years' use of progestin-only contraceptives is also associated with a slight increased risk for invasive cervical cancer (relative risk: 1.22 [95% CI: 1.01‑1.46) (3). It remains unclear whether the association between hormonal contraceptive use and cervical cancer is driven by its effect(s) on carcinogenesis and/or on upstream events such as HPV prevalence, acquisition and persistence.

The current understanding of the epidemiologic relationship between hormonal contraceptive use and HPV infection is limited in several aspects. Previous studies were conducted in different countries that had varied baseline HPV prevalence and screening practice (7). Results have been mixed across studies. Null associations or associations in both directions have been reported (8-32). Several studies were limited by confounding

by Pap smear history. Duration of hormonal contraceptive use, which has been shown to relate to risk of HPV prevalence or cervical lesion development (10, 26), was not adequately assessed in many previous studies. In addition, current published data on the association between hormonal contraceptive use and HPV infection have mostly been focused on the use of oral contraceptives (OCs) among women below the age of 40 years, or across a broad age spectrum with relatively small proportion of the study population in the older age group. Furthermore, few studies examined the influence of hormonal contraceptives other than OC or COC on HPV infection (8, 10, 14, 25, 27, 28), or cervical neoplasia (3, 33).

The goal of this study was to evaluate the cross-sectional association between hormonal contraceptive use and HPV prevalence among pre- and perimenopausal women aged over 35 years who have normal cytology in the U.S. Associations were assessed in reference to the duration and recency of hormonal contraceptive use including oral contraceptives and progestin-only contraceptives.

MATERIALS AND METHODS

Study design

Data were obtained from an observational prospective study assessing the natural history of HPV during the perimenopausal transition. Women aged 35 to 60 years were recruited from gynecological clinics associated with the Johns Hopkins Medical Institutions in Baltimore, Maryland. Exclusion criteria for the parent study included the following: (i) history of hysterectomy, organ transplant or HIV infection, (ii) currently pregnant, (iii)

unable to provide informed consent, (iv) unwilling to provide contact information, (v) non-English speaking. Participants were enrolled at a baseline study visit, and were followed every 6 months for a planned period of 2 years. The study protocols were reviewed and approved by the Institutional Review Board at Johns Hopkins Bloomberg School of Public Health, Baltimore, MD.

Standardized questionnaires were administered by trained interviewers at baseline and follow-up visits. Parameters assessed were either shown to be associated with risk of HPV infection, cervical cancer risk or age-related decline in immune function to allow confounding control in analyses. Information was collected on (i) use of hormonal contraceptives including duration of use, time since last use, indications of use and brand names, (ii) demographic information, (iii) sexual behavioral characteristics, (iv) reproductive history, (v) cigarette and alcohol use, (vi) use of medications other than hormonal contraceptives, (vii) history of sexually transmitted infections, (viii) use of medications other than hormonal contraceptives.

Physical examination and specimen collection

A comprehensive physical examination and pelvic examination were performed at baseline visit. A cervical secretion specimen, liquid-based Pap smear and cervical swab (Digene HPV sampler kit) were obtained. Cervical secretions were collected using a Merocel ophthalmic sponge which was placed at 6 o'clock of the cervical os for 30 seconds. The collected cervical sample was stored at -80 ^{o}C until testing.

Liquid-based Pap smears were performed where clinically indicated. Results of pap smears were abstracted from medical records and the cytological abnormalities were classified based on the 2001 Bethesda classification scheme [normal, inflammation, ASCUS (aytpical squamous cells – undetermined significance), LSIL (low grade squamous intraepithelial lesion), HSIL (high grade squamous intraepitheilial lesion)].

HPV DNA type-specific testing was done on cervical swab samples. DNA was extracted using the QIAamp DNA Blood Kit (Qiagen, Courtaboeuf, France) according to manufacturer's instructions with modification. After extraction, 8 µls of DNA (4% of total volume of extracted DNA) was amplified using the PGMY09/11 L1 consensus primer system, which amplifies with high efficiency over 40 HPV genotypes known to infect the genital tract. Genotype discrimination was performed by hybridizing 40 µl of the PCR product to a membrane with immobilized probes targeting 37 HPV genotypes (HPV Linear Array Roche Molecular Systems, Roche Diagnostics, Indianapolis, IN).

Measures and definition of terms

Primary exposure of interest

The primary exposure of interest was self-reported use of hormonal contraceptives at baseline, which include oral formulations, injections, dermal patches, intrauterine or intravaginal devices containing or releasing estrogen and/or progestin derivatives which are approved by FDA for contraception. Exposure variables were categorized by recency

and duration of use based on the observed distribution of outcome responses. Time since last use was categorized into "never user," "current user," "<5 years ago", "6-10 years ago," ">10 years ago." Those who reported use within the last month were categorized as current users. Duration of use was examined in categories of "never user," "≤ 1 year," "1.1-5 years," and ">5 years." A variable combining both current and former use with cumulative duration of exposure was also created to study the joint effects of recency and duration of hormonal contraceptive use. Long-term and short-term uses were defined as use for >5 and ≤5 years respectively.

Secondary analyses were done with further categorizations of the hormonal contraceptive use: (i) oral contraceptives (OC), (ii) progestin-only contraceptives including progestin-only pills (minipills), injectable contraceptives [depot medroxyprogesteroneacetate (DMPA)], etonogestrel single-rod implant, and intrauterine contraceptive containing levonorgestrel such as Mirena. The category of oral contraceptives included participants reporting the use of "combined oral contraceptives (COC)", medication brand names that can be classified pharmaceutically as COC, as well as the "pill(s)." Given the generally accepted notion that "the pill" usually refers to combined oral contraceptive pill (34, 35), the category of OC likely represents mostly COC users. Sensitivity analyses were done categorizing only those who were able to report the specific COC medication brand names as COC users for comparison.

Outcome of interest

The primary outcome of interest was detection of any type-specific HPV DNA in the cervical samples collected at baseline, which include 37 HPV types 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 82 subtype (IS39), 83, 84, 89 (CP6108).

Secondary outcomes of interest included detection of any high-risk HPV type, which was defined as one of the following HPV types identified by the International Collaboration of Epidemiological Studies of Cervical Cancer as oncogenic (HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68) (36).

Other measures

For this cross-sectional analysis, we focused on the baseline visit and selected women who were pre- and perimenopausal (reproductive and menopausal transition) as defined by the criteria proposed by the Stages of Reproductive Aging Workshop (40). Briefly, premenopause/reproductive age was defined as having overall regular menstrual periods or subtle changes of menstrual flow. Perimenopause/menopausal transition was defined as reporting irregular menstrual periods in the last 12 months with last menstrual period <12 months ago. Postmenopause was defined as >12 months since last reported menstrual period (40).

Abnormal Pap smear was defined as having cytologic diagnoses of ASCUS, LSIL and HSIL. Time since last abnormal Pap smear was categorized into “never,” “<1 year ago,” “2-5 years ago,” and “>5 years ago,” Sexually transmitted infections included self-

reported genital chlamydia, gonorrhea, syphilis, herpes, trichomoniasis, chancroid, warts. Other variables were categorized based on frequency of distribution of outcomes of interest. Age was categorized into "35-39 years," "40-44 years," "45-49 years," and "≥50 years." Race was categorized as "Caucasian," "African American," and "others" which included American Indians, Pacific Islanders, Asians and those who were unidentified. Number of livebirths was categorized into "0-1," "2," and ">2." Lifetime number of sexual partners was categorized into "1," "2-4," and "≥5." Recent sex exposure was described by a variable on sexual activity in the past 6 months: "no sex," "have sex but no new sex partner," and "have sex with new sex partner."

Statistical Analysis

Analysis population

The population eligible for analysis included participants who were pre- and perimenopausal who did not report use of exogenous hormones other than hormonal contraceptives (n=618). Among these participants, the following subjects were excluded from analyses: (i) inadequate baseline HPV DNA testing result (n=1, 0.2%), (ii) missing information on hormonal contraceptive use (n=22, 3.6%), (iii) missing responses on recent history of other sexually transmitted infections (n=4, 0.7%), (iv) missing information on sexual behavioral risk factors (n=4, 0.7%), (v) abnormal Pap smear (n=35) or unclear Pap smear status (n=19) at baseline (total n=54, 8.7%), (vi) unclear time since last abnormal Pap smear (n=4, 0.7%). The exclusions resulted in a total of 530 subjects included in final analyses (85.8% of eligible population).

Exploratory data analysis

Variables assessed in this study dataset were categorically coded. Distribution of demographic, sexual behavioral characteristics, primary outcome and exposure characteristics in the study population were evaluated using Pearson's Chi-square or Fisher's exact test if cell count was <5.

Univariable modeling

Characteristics found to be of significant association ($p<0.05$) based on Chi-square tests were further assessed by univariate prevalence ratios (PR). PRs estimated by a generalized linear model with Poisson function with robust variance were used to evaluate the strength of association between a given risk factor and detection of any or high-risk HPV. Characteristics which were shown in previous literature to be risk factors for any and/or high-risk HPV infections; and/or yielded statistically significant association or potential statistically significant association ($p<0.10$) in univariable models were considered in multivariable models.

Multivariable modeling

Selection of predictors was done by forward and backward selection of factors with cut-off P value at 0.05. Covariates that showed >10% change in the effect size of the primary exposure and outcome association were kept in the final model. If supported by literature to be important risk factors for HPV progression, predictors which did not yield statistically significant association were also included in the final model based on

epidemiologic considerations even though the final model was not the most parsimonious model with Akaike's information criterion values.

Sensitivity analyses

For secondary analyses with each type of hormonal contraceptive exposure, 2 sets of analyses with 2 different reference groups were created for comparison: (i) one reference group included only those who never used any hormonal contraceptives, and (ii) the other reference group comprised never users of any hormonal contraceptives as well as individuals who never used that particular type of hormonal contraceptives being assessed.

Missing data was included as a response in the variables that had unclear or missing data, which included recent history of other sexually transmitted infections, sexual behavioral risk factors and Pap smear status. Final models were also constructed with these variables including these missing responses to assess the influence of missing data. Results and conclusions were unchanged with exclusion or inclusion of missing responses.
All analyses were done using Stata/SE version 11 (StataCorp, College Station, TX).

RESULTS

Baseline characteristics of study population and association with HPV prevalence

A total of 530 participants were included in data analyses. The mean age was 44.2 years (SD: 5.4 years; range: 35-54 years). Over 70% of the participants were Caucasian, and

more than half attained college or higher level of education. Majority of women had 2 or more livebirths (67.8%) and were sexually active at baseline (83.7%). Most subjects had multiple lifetime sex partners (88.9%). Over 60% participants claimed having more than 5 sex partners but only 2.8% reported new sexual partners in the past 6 months. Most subjects were categorized as premenopausal based on their days of menstrual cycle (75.6%). About half of the population (45.5%) had abnormal Pap smear in the past, with 2.1% having the diagnosis within the past year (Table 3.1).

Sixteen percent (16.4%, n=87) of the study population had HPV detected, of whom 23.0% (n=20) had infection with multiple HPV types. The overall prevalence of high-risk (HR) HPV was 6.4% (Table 3.1). The most common HR-HPV type detected was HPV 52, followed by HPV 16, 39 and 18. HPV 83, 62, and 55 were the most common low-risk HPV types detected in the study population (Figure 3.1).

Higher HPV prevalence was observed among women reporting recent diagnoses (≤6 months ago) of other sexually transmitted infections, having abnormal Pap smear in the past year, reporting new sexual partner in the past 6 months or having more than 5 lifetime sex partners. Women who were single had more prevalent HPV infections detected compared with currently married women. Subjects younger than 39 years had more prevalent HR-HPV infections diagnosed compared with women aged ≥ 40 years (Table 3.1).

After controlling for other risk factors for HPV, our final model showed that having new sexual exposure in the past 6 months but not multiple lifetime sex partners was statistically significantly associated with prevalent HPV infection (Table 3.3). Other independent predictors for HPV prevalence included marital status, diagnosis of abnormal Pap smear within the past year, and recent history of other sexually transmitted infections (Table 3.3).

Baseline hormonal contraceptive use in study population

Most women had used hormonal contraceptives (HCs) at some point in their lives (89.4%). Over half (54.4%) of women last used HCs more than 5 years ago (Table 3.2). Thirty-six percent (36.3%) of the study population had cumulative exposure to HCs for more than 5 years (n=192). 21.1% (n=112) were currently using HCs at baseline (Table 3.2). Overall 8.9% (n=47) reported current use of progestin-only contraceptives (POCs). More than half of them were Mirena users (55.3%, n=26), with the rest being progestin pill users (n=18) and DMPA users (n=3).Among those who were long-term (> 5 years) ever users of POCs (n=16) (Table 3.2), majority of them were either users of DMPA (n=9) or Mirena (n=5).

Over half of current HC users were younger than 44 years (67.0%). Forty percent of current HC users were either nulliparous or had only 1 livebirth. Close to half (42.5%) of those who used HCs solely for gynecologic conditions were current HC users.

Association between hormonal contraceptive use and HPV detection at baseline

Hormonal contraceptive use in general:

No significant association was found between HPV detection and the duration or recency of overall HC use in univariable or multivariable analyses (Table 3.3, Table 3.4).

Progestin-only contraceptive use:

Significantly higher proportions of long-term (>5 years) POC users (50%) were HPV-positive than those who were short-term (20.8%) or never users (14.8%) (P=0.01) (Table 3.2). More current users of POCs were positive for HPV (27.7%) than never users (14.3%) (P=0.02) (Table 3.2).

Compared with never POC users, more than 5 years of POC use was associated with nearly 3-fold increased HPV prevalence after controlling for other independent risk factors including having more than 5 lifetime sex partners, reporting recent new sex partner, being single or divorced, being diagnosed with other sexually transmitted infections in the past 6 months, and having abnormal pap smears within the past year [adjusted prevalence ratio (aPR): 3.16 (95% CI: 1.82-5.48)] (Table 3.5). Prevalence of HR-HPV was also increased with long-term use of POCs compared with never POC users [aPR: 4.26 (95% CI: 1.6-11.3)](Table 3.5). Current POC use was positively associated with HR-HPV (aPR: 2.44 (0.99-5.99) and any HPV (aPR: 1.58 (0.94-2.65) with the estimates bordering on statistical significance after controlling for other risk factors (Table 3.6).

When the reference group became never users of any HCs, more than 5 years' use of POC was still found to associate with nearly 3-fold increase of any HPV prevalence after adjusting for other risk factors. Association with HR-HPV prevalence did not achieve statistical significance between long duration of use and HR-HPV prevalence (Appendix. Appendix Tables 3.1-3.2).

Oral contraceptive use:

Neither duration nor recency of OC use yielded statistically significant association with detection of any or HR-HPV at baseline after controlling for confounders, regardless of the reference group being never OC users or never HC users. (Table 3.7-3.8; Appendix Table 3.3-3.4). Similar findings were observed among users who could confirm the brand names of the COC they used (Appendix Tables 3.5-3.6).

DISCUSSION

Our study found that recency and duration of overall hormonal contraceptive use was not associated with any or HR-HPV detection in reproductive women over the age of 35 years. Long-term (>5 years) use of POCs was observed to associate with increased risk of prevalent HPV infection after controlling for other risk factors. We did not detect significant association of OC or COC use with HPV prevalence in our study population.

Previous findings on the effects of OC or COC on HPV infection have been mixed (8-32). Reported associations differ due to heterogeneity in study locations, measure metrics and study designs. Our study results are consistent with those of pooled analyses to date,

which reveal neither a strong positive or negative association between OC use and HPV prevalent infection even after considering duration of OC use and confounding control of sexual activity (24, 26). Past studies that showed positive association between OC and HPV infection were mostly conducted in young women below the age of 40 years (8, 10, 13, 16, 21, 22, 29, 30-32), who were likely to have different sexual behavioral profiles than our study population of older females (41). Furthermore, pharmacokinetic differences have been reported on COC and ethynl estradiol in human subjects with different ethnic backgrounds (42-45). The bioavailable doses of estrogen derivatives may therefore be greater in certain populations than others. If dosage of OC or COC has an impact on HPV infection, the different bioavailabilities of exogenous estrogen derivatives may result in positive association observed between OC or COC and HPV in certain ethnic populations but not in others.

Our finding on POC use and HPV infection is also consistent with recent studies conducted among younger women in the U.S., which reported positive associations between injectable contraceptive use and HPV prevalence (25, 27, 28). Prospective studies examining the effects of POCs on HPV have been relatively scarce with mixed findings (8, 14, 27). A recent study did not find significant association between DMPA use and HPV prevalence, persistence or incidence in Thai young females (8). In the U.S., one large cohort study showed that 6-month persistence of HPV infection was mildly elevated among users of injectable contraceptives [OR: 1.15 (95% CI: 1.01-1.32)] who had low-grade or equivocal cytological abnormalities (14). Another U.S. study reported positive association between ≥1-year use of DMPA and oncogenic HPV infection after

controlling for sexual behavioral factors (adjusted OR: 7.3; 95% CI, 1.5-35.5)(27). Type-specific HPV persistence, however, was not found in the latter study (27), suggesting that DMPA use might be associated more with new HPV detection than persistence in that study population.

DMPA use was postulated to promote HPV infection as it was found to associate with atrophic cervical epithelium as well as different degrees of nuclear atypia (46, 47). The compromise of the epithelial barrier may facilitate the seeding and establishment of viral agents such as HPV. In addition, DMPA use was found to associate with acquisition of chlamydial infection (48-51), which is proposed to be a cofactor for HPV infection (48-50).

Female sex hormones have been suggested to influence host immunity and viral replication (52). Progesterone has been shown to inhibit cytotoxic T lymphocyte activity and perforin expression on T cells (52). Progesterone also has impact on cytokine production that generally skews towards Th2 response (52, 53). In addition, progesterone and glucocorticoid hormones have been found to increase HPV viral mRNA and significantly stimulate HPV viral replication in vitro (54, 55).

Cumulative exposure to POCs may also be influenced by dosages of progesterone administration. Previous study noted that while DMPA (a high-dose POC) was associated with increased HPV persistence, use of Norplant (a low-dose POC) was not (14). Our study was limited in sample size and statistical power to examine the respective associations of different kinds of POC use with HPV prevalence, which is an area warranting further investigation in follow-up studies.

Our study has a number of strengths. Extensive data were collected on the status, types, recency and duration of hormonal contraceptive use. This allowed more specific characterization of exogenous sex hormone exposure as well as its association with HPV infection. Detailed information was also obtained on sexual behavior and other risk factors to assess covariates that may confound the associations between HPV prevalence and hormonal contraceptive use. Menopausal stages were determined biologically based on days of menstrual cycle which lent relevance to the actual endogenous female sex hormonal levels in the subjects. In addition, our study population had a baseline racial demographic that was generally representative of the U.S. population in this age range (56, 57). Lifetime and recent sexual behavioral characteristics also correlated well with recent estimates of the U.S. national sample in this age group (41), thus enhancing the relevance of our findings to the population from which they were sampled.

Our study also has its limitations. Participants in this study were recruited from women presenting to gynecological clinics who were routinely screened with Pap smears. The generalizability of our results to the general older female population may thus be limited. The HR-HPV prevalence in our population (6.4%) was comparable to the multi-site estimate (9%) reported by Centers for Disease Control and Prevention for women aged over 30 years who had normal cervical cytology and presented to clinics for cervical screening in the U.S (58). The prevalence findings in such clinic population, however, varied from some other population estimates due to differences in study demographics and laboratory protocols in detecting HPV DNA. Relative to our study estimate, a recent

meta-analysis done by Bruni et al found an almost 3-fold lower level of overall HPV prevalence among North American women with normal Pap smears in this age group (7). The difference may be attributable to the inclusion of a large study composing of a significantly higher proportion of Asians compared with national samples (7, 59), and Asians were generally found to have lower HPV prevalence than other ethnic groups (7). Our HR-HPV prevalence was lower than that (27% for age 30- 59 years) found from the National Health and Nutrition Examination Survey (NHANES), which oversampled African Americans and Hispanics and used a more liberal definition of HR-HPV (60, 61). Such difference in prevalence estimate is also due to the ability of the laboratory protocol used in the NHANES study to detect very low levels of HPV DNA (60). Supposing that our study was less sensitive in HPV detection, our findings may have underestimated the magnitude of the actual outcome prevalence. False negatives and type II error are higher, thereby limiting our power to detect a potential association if it exists.

The directionality of the association observed in our study is, however, still expected to be applicable to the general population, assuming there was minimal selection bias and unmeasured or residual confounding. One of the implications of assessing hormonal contraceptive use as determinant of HPV detection was to improve testing-associated counseling of women who were being screened for cervical cancer. Given that, our study population was most relevant to our target population, and the results of our study were generalizable to the target population.

We were limited on self-reported data on hormonal contraceptive use. There was possibility of under-report of use as well as differential recall of use related to underlying

conditions. These might introduce misclassification of exposure which would bias the estimates of associations. However, previous studies comparing self-reported contraceptive use to clinical records in women aged 20 to 42 years have shown relatively high agreement (61), especially with regard to the duration and hormonal composition of current and recent use (62, 63). The amount of misclassification introduced by discrepancies in self-report of recent use of hormonal contraceptives would thus be relatively small, which would likely not obscure the directionality of associations seen in this study.

Our study was also limited on self-report for information on sexual risk behaviors, making the observed associations subject to potential unmeasured and residual confounding. This study was conducted in an older age group who were found to be generally less sexually active than younger females (41). Our data also showed that few reported new recent sex exposure prior to baseline (2.8%). The confounding effect of unmeasured sexual behavior on the associations assessed would therefore likely be reduced compared to the younger populations assessed in previous studies.

In conclusion, we found that long-term use of POCs was associated with increased prevalence of HPV infection in pre- and perimenopausal women above the age of 35 years in the U.S. No significant association was observed between OC use and HPV prevalence in this population. Since prevalence is determined by both incidence and duration of diseases, longitudinal studies are needed to better determine the long-term effects of POC and OC use on HPV infection in women of this age group.

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TABLES AND FIGURES

Table 3.1. Baseline characteristics of study population (N=530)

Characteristics at baseline	Total N=530 (col %)[a]	Any HPV+ n= 87 (16.4%) (row %)	P	HR-HPV +[b] n=34 (6.4%) (row %)	P
Demographic characteristics					
Age (years)			0.12		**0.02**
35-39	126 (23.8)	29 (23.0)		15 (11.9)	
40-44	148 (27.9)	19 (12.8)		10 (6.8)	
45-49	157 (29.6)	22 (14.0)		6 (3.8)	
≥50	99 (18.7)	17 (17.2)		3 (3.0)	
Race			0.36		0.68
Caucasian	392 (74.0)	60 (15.3)		23 (5.9)	
African American	99 (18.7)	21 (21.2)		8 (8.1)	
Others[c]	39 (7.4)	6 (15.4)		3 (7.7)	
Education status			**0.01**		0.12
High school	84 (15.9)	8 (9.5)		4 (4.8)	
Post-high school	122 (23.0)	30 (24.6)		13 (10.7)	
College graduate	166 (31.3)	30 (18.1)		11 (6.6)	
Post-college	158 (29.8)	19 (12.0)		6 (3.8)	
Marital status			**<0.001**		**0.005**
Married	339 (64.0)	35 (10.3)		13 (3.8)	
Single	107 (20.2)	33 (30.8)		12 (11.2)	
Divorced/separated/widowed	84 (15.9)	19 (22.6)		9 (10.7)	
Current smoker			0.71		0.39
No	475 (89.6)	77 (16.2)		29 (6.1)	
Yes	55 (10.4)	10 (18.2)		5 (9.1)	
Parity			**0.02**		0.73
0 or 1	170 (32.1)	39 (22.9)		13 (7.7)	
2	124 (23.4)	14 (11.3)		7 (5.7)	
>2	235 (44.4)	34 (14.5)		14 (6.0)	
Sexual behavioral characteristics					
Lifetime number of sexual partners			**0.02**		0.67

≤2	59 (11.1)	5 (8.5)		3 (5.1)	
3-4	134 (25.3)	14 (10.5)		6 (4.5)	
5-6	215 (40.6)	43 (20.0)		16 (7.4)	
>10	122 (23.0)	25 (20.5)		9 (7.4)	
Sexual activity in last 6 months			**<0.001**		**<0.001**
No sex	86 (16.2)	12 (14.0)		5 (5.8)	
Have sex but no new sexual partner	429 (80.9)	67 (15.6)		22 (5.1)	
Have sex with new sexual partner	15 (2.8)	8 (53.3)		7 (46.7)	
Last menstrual period (days)			0.18		0.70
1-14	227 (42.8)	46 (20.3)		14 (6.2)	
15-28	174 (32.8)	26 (15.0)		13 (7.5)	
28-40	34 (6.4)	4 (11.8)		3 (8.8)	
40-365	95 (17.9)	11 (11.6)		4 (4.2)	
Ever undergone colposcopy			0.60		0.06
No	425 (80.2)	68 (16.0)		23 (5.4)	
Yes	105 (19.8)	19 (18.1)		11 (10.5)	
Time since last abnormal pap smear			0.06		0.12
Never had abnormal pap smear	289 (54.5)	43 (14.9)		13 (4.5)	
<1 year ago	11 (2.1)	5 (45.5)		2 (18.2)	
2-5 years ago	59 (11.1)	11 (18.6)		4 (6.8)	
≥6 years ago	171 (32.3)	28 (16.4)		15 (8.8)	
Diagnosed with other sexually transmitted infections in the past 6 months [d]			**0.03**		0.10
No	517 (94.5)	80 (15.5)		32 (6.2)	
Yes	13 (5.5)	7 (53.9)		2 (15.4)	

[a.] Percentages or numbers in categories may not add up to 100% or the total number due to missing data.

[b.] HR-HPV types: oncogenic HPV as defined as types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 (IARC 2007).

[c.] Other categories include American Indian (n=2), Pacific Islander (n=2), Asian (n=16), unidentified (n=9).

[d.] Other sexually transmitted infections included genital chlamydia (n=1), gonorrhea (n=0), herpes (n=9), syphilis (n=0), trichomoniasis (n=3), chancroid (n=0), warts (n=0).

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Table 3.2. Baseline hormonal contraceptive use and HPV detection in study population (N=530)

Characteristics at baseline		Total N=530 (col %)[a]	Any HPV+ n= 87 (16.4%) (row %)	P	HR-HPV +[b] n=34 (6.4%) (row %)	P
Hormonal contraceptives: Total lifetime exposure						
Duration of use by recency	**Years**			0.58		0.19
Never used any HC		56 (10.6)	7 (12.5)		4 (7.1)	
Current	≤5	82 (15.5)	16 (19.5)		6 (7.3)	
	>5	30 (5.9)	8 (25.8)		4 (12.9)	
Former	≤5	179 (33.8)	26 (14.5)		12 (6.7)	
	>5	161 (30.4)	26 (16.2)		5 (3.1)	
Unclear duration		22 (4.0)	4 (19.1)		3 (14.3)	
Time since last use				0.35		0.57
Never user		56 (10.6)	7 (12.5)		4 (7.1)	
Current user		112 (21.1)	24 (21.4)		10 (8.9)	
Last use ≤ 5 years ago		74 (14.0)	11 (14.9)		2 (2.7)	
Last use 6-10 years ago		63 (11.9)	13 (20.6)		4 (6.4)	
Last use >10 years ago		225 (42.5)	32 (14.2)		14 (6.2)	
Use of any progestin-only contraceptives						
Duration of use	**Years**			**0.01**		**0.001**
Never used any progestin-only contraceptives		440 (83.0)	63 (14.3)		24 (5.5)	
	≤5	72 (13.6)	15 (20.8)		5 (6.9)	
	>5	16 (3.0)	8 (50.0)		4 (25.0)	
Cannot be determined		2 (0.4)	1 (50.0)		1 (50.0)	
Time since last use				**0.02**		0.11
Never used any progestin-only contraceptives		440 (83.0)	63 (14.3)		24 (5.5)	
Current user		47 (8.9)	13 (27.7)		6 (12.8)	
Past user		43 (8.1)	11 (25.6)		4 (9.3)	
Use of oral contraceptives						

Duration of use	**Years**			0.43		0.10
Never used any oral contraceptives		95 (17.9)	21 (22.1)		10 (10.5)	
	≤5	211 (39.8)	32 (15.2)		14 (6.6)	
	>5	197 (37.2)	30 (15.2)		7 (3.6)	
Cannot be determined		27 (5.1)	4 (14.8)		3 (11.1)	
Time since last use				0.25		0.19
Never used any oral contraceptives		95 (17.9)	21 (22.1)		10 (5.1)	
Current user		63 (11.9)	10 (15.9)		4 (6.4)	
Past user		372 (70.2)	56 (15.1)		20 (5.4)	

[a.] Percentages or numbers in categories may not add up to 100% or the total number due to missing data.
[b.] HR-HPV types: oncogenic HPV as defined as types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 (IARC 2007).

Table 3.3.Association between HPV detection and total exposure to any hormonal contraceptives (N=530)

Characteristics		Total N N(col %)	Positive for any HPV n=87 (16.4%) Any HPV+ n(row %)	Unadjusted PR (95% CI)	Adjusted PR[a] (95% CI)	Positive for HR-HPV n=34 (6.4%) HR-HPV+ n(row %)	Unadjusted PR (95% CI)	Adjusted PR[a] (95% CI)
Use of hormonal contraceptives								
Total duration of use	**Years**							
Never user		56 (10.6)	7 (12.5)	Ref	Ref	4 (7.1)	Ref	Ref
Current user	≤5	82 (15.5)	16 (19.5)	1.56 (0.69-3.55)	1.18 (0.52-2.71)	6 (7.3)	1.02 (0.30-3.47)	0.87 (0.25-3.06)
	>5	30 (5.9)	8 (25.8)	2.06 (0.83-5.16)	1.59 (0.65-3.87)	4 (12.9)	1.81 (0.48-6.73)	0.92 (0.20-4.27)
Past user	≤5	179 (33.8)	26 (14.5)	1.16 (0.53-2.53)	0.96 (0.45-2.03)	12 (6.7)	0.94 (0.31-2.80)	0.83 (0.28-2.41)
	>5	161 (30.4)	26 (16.2)	1.29 (0.59-2.81)	1.27 (0.59-2.73)	5 (3.1)	0.43 (0.12-1.56)	0.38 (0.11-1.29)
Unclear duration		22 (4.0)	4 (19.1)	1.52 (0.50-4.68)	1.07 (0.39-2.94)	3 (14.3)	2.00 (0.49-8.21)	1.24 (0.32-4.85)
Time from last menstrual period	**Days**							
	1-14	227 (42.8)	46 (20.3)	Ref	Ref	14 (6.2)	Ref	Ref
	15-28	174 (32.8)	26 (15.0)	0.74 (0.48-1.14)	0.70 (0.45-1.09)	13 (7.5)	1.21 (0.58-2.51)	1.18 (0.58-2.41)
	28-40	34 (6.4)	4 (11.8)	0.58 (0.22-1.51)	0.71 (0.26-1.95)	3 (8.8)	1.43 (0.43-4.73)	1.74 (0.50-6.13)
	40-365	95 (17.9)	11 (11.6)	0.57 (0.31-1.06)	0.56 (0.30-1.03)	4 (4.2)	0.68 (0.23-2.02)	0.68 (0.24-1.97)
Lifetime number of sexual partners								
	≤2	59 (11.1)	5 (8.5)	Ref	Ref	3 (5.1)	Ref	Ref
	3-4	134 (25.3)	14 (10.5)	1.23 (0.46-3.27)	1.08 (0.41-2.87)	6 (4.5)	0.88 (0.23-3.40)	0.65 (0.18-2.35)
	≥5	337 (63.6)	68 (20.2)	**2.38 (1.01-5.66)**	1.56 (0.65-3.75)	25 (7.4)	1.46 (0.45-4.68)	0.81 (0.25-2.67)
Sexual activity in last 6 months								
No sex		86 (16.2)	12 (14.0)	Ref	Ref	5 (5.8)	Ref	Ref
Have sex but no new sexual partner		429 (80.9)	67 (15.6)	1.12 (0.63-1.98)	1.69 (0.96-2.96)	22 (5.1)	0.88 (0.34-2.27)	1.11 (0.43-2.92)
Have sex with new sexual partner		15 (2.8)	8 (53.3)	**3.82 (1.89-7.75)**	**3.25 (1.51-7.03)**	7 (46.7)	**8.03 (2.93-22.02)**	**6.24 (2.05-19.00)**
Marital status								
Married		339 (64.0)	35 (10.3)	Ref	Ref	13 (3.8)	Ref	Ref
Single		107 (20.2)	33 (30.8)	**2.99 (1.96-4.56)**	**2.83 (1.82-4.43)**	12 (11.2)	**2.92 (1.38-6.22)**	2.01 (0.84-4.85)
Divorced/separated/widowed		84 (15.9)	19 (22.6)	**2.19 (1.32-3.63)**	**2.01 (1.20-3.37)**	9 (10.7)	**2.79 (1.23-6.32)**	2.15 (0.84-5.49)

Time since last abnormal pap smear							
Never	289 (54.5)	43 (14.9)	Ref	Ref	13 (4.5)	Ref	Ref
< 1 year ago	11 (2.1)	5 (45.5)	**3.05 (1.51-6.18)**	**2.60 (1.35-5.02)**	2 (18.2)	**4.04 (1.03-15.79)**	3.29 (0.81-13.27)
2-5 years ago	59 (11.1)	11 (18.6)	1.25 (0.69-2.28)	0.94 (0.52-1.72)	4 (6.8)	1.51 (0.51-4.47)	1.08 (0.35-3.34)
≥ 6 years ago	171 (32.3)	28 (16.4)	1.10 (0.71-1.70)	0.81 (0.53-1.24)	15 (8.8)	1.95 (0.95-4.00)	1.54 (0.75-3.16)
Been diagnosed with other STIs in the past 6 months							
No	517 (94.5)	80 (15.5)	Ref	Ref	32 (6.2)	Ref	Ref
Yes	13 (5.5)	7 (53.9)	**3.48 (2.02-5.99)**	**3.47 (1.94-6.21)**	2 (15.4)	2.48 (0.66-9.30)	2.02 (0.54-7.63)

[a] Adjusted for days from last menstrual period, number of lifetime sex partners, acquisition of new sex partner in last 6 months, marital status, time since last abnormal pap smear, history of other sexually transmitted diseases in the last 6 months.

Table 3.4. Association between HPV detection and last time of use of hormonal contraceptives (N=530)

Characteristics	Total N N(col %)	Positive for any HPV n=87 (16.4%) Any HPV+ n(row %)	Unadjusted PR (95% CI)	Adjusted PR[a] (95% CI)	Positive for HR-HPV n=34 (6.4%) HR-HPV+ n(row %)	Unadjusted PR (95% CI)	Adjusted PR[a] (95% CI)
Last time of use of any hormonal contraceptives							
Never user	56 (10.6)	7 (12.5)	Ref	Ref	4 (7.1)	Ref	Ref
Current user	112 (21.1)	24 (21.4)	1.71 (0.78-3.74)	1.29 (0.59-2.83)	10 (8.9)	1.25 (0.41-3.81)	0.88 (0.27-2.90)
Last use ≤ 5 years ago	74 (14.0)	11 (14.9)	1.19 (0.49-2.87)	0.83 (0.35-1.97)	2 (2.7)	0.38 (0.07-2.00)	0.28 (0.05-1.50)
Last use 6-10 years ago	63 (11.9)	13 (20.6)	1.65 (0.71-3.85)	1.52 (0.65-3.59)	4 (6.4)	0.89 (0.23-3.39)	0.78 (0.20-3.06)
Last use >10 years ago	225 (42.5)	32 (14.2)	1.14 (0.53-2.44)	1.07 (0.51-2.23)	14 (6.2)	0.87 (0.30-2.55)	0.79 (0.27-2.29)

[a] Adjusted for days from last menstrual period, number of lifetime sex partners, acquisition of new sex partner in last 6 months, marital status, time since last abnormal pap smear, history of other sexually transmitted diseases in the last 6 months.

Table 3.5. Association between HPV detection and total exposure to any progestin-only contraceptives, with never users of progestin-only contraceptives as reference group (N=530)

Characteristics		Total N N(col %)	Positive for any HPV n=87 (16.4%) Any HPV+ n(row %)	Unadjusted PR (95% CI)	Adjusted PR[a] (95% CI)	Positive for HR-HPV n=34 (6.4%) HR-HPV+ n(row %)	Unadjusted PR (95% CI)	Adjusted PR[a] (95% CI)
Use of progestin-only contraceptives								
Total duration of use	**Years**							
Never user		440 (83.0)	63 (14.3)	Ref	Ref	24 (5.5)	Ref	Ref
Ever user								
	≤5	72 (13.6)	15 (20.8)	1.46 (0.88-2.41)	1.17 (0.70-1.96)	5 (6.9)	1.27 (0.50-3.23)	1.32 (0.52-3.35)
	>5	16 (3.0)	8 (50.0)	**3.49 (2.03-5.99)**	**3.16 (1.82-5.48)**	4 (25.0)	**4.58 (1.80-11.67)**	**4.26 (1.60-11.30)**
Unclear duration		2 (0.4)	1 (50.0)	3.49 (0.86-14.25)	2.06 (0.73-5.77)	1 (50.0)	**9.17 (2.17-38.72)**	**5.46 (1.07-27.83)**

[a] Adjusted for days from last menstrual period, number of lifetime sex partners, acquisition of new sex partner in last 6 months, marital status, time since last abnormal pap smear, history of other sexually transmitted diseases in the last 6 months.

Table 3.6. Association between HPV detection and recency of any progestin-only hormonal contraceptive use, with never users of progestin-only contraceptives as reference group (N=530)

Characteristics	Total N N(col %)	Positive for any HPV n=87 (16.4%) Any HPV+ n(row %)	Unadjusted PR (95% CI)	Adjusted PR[a] (95% CI)	Positive for HR-HPV n=34 (6.4%) HR-HPV+ n(row %)	Unadjusted PR (95% CI)	Adjusted PR[a] (95% CI)
Use of progestin-only contraceptives							
Last time of use							
Never user	440 (83.0)	63 (14.3)	Ref	Ref	24 (5.5)	Ref	Ref
Current user	47 (8.9)	13 (27.7)	**1.93 (1.15-3.24)**	1.58 (0.94-2.65)	6 (12.8)	**2.34 (1.01-5.44)**	2.44 (0.99-5.99)
Past user	43 (8.1)	11 (25.6)	**1.79 (1.02-3.13)**	1.51 (0.88-2.61)	4 (9.3)	1.71 (0.62-4.69)	1.65 (0.69-3.96)

[a] Adjusted for days from last menstrual period, number of lifetime sex partners, acquisition of new sex partner in last 6 months, marital status, time since last abnormal pap smear, history of other sexually transmitted diseases in the last 6 months.

Table 3.7. Association between HPV detection and total exposure to any oral contraceptives, with never users of oral contraceptives as reference group (N=530)

Characteristics		Total N N(col %)	Positive for any HPV n=87 (16.4%) Any HPV+ n(row %)	Unadjusted PR (95% CI)	Adjusted PR[a] (95% CI)	Positive for HR-HPV n=34 (6.4%) HR-HPV+ n(row %)	Unadjusted PR (95% CI)	Adjusted PR[a] (95% CI)
Use of oral contraceptives								
Total duration of use	**Years**							
Never user		95 (17.9)	21 (22.1)	Ref	Ref	10 (10.5)	Ref	Ref
Ever user	≤5	211 (39.8)	32 (15.2)	0.69 (0.42-1.13)	0.69 (0.43-1.11)	14 (6.6)	0.63 (0.29-1.37)	0.63 (0.29-1.37)
	>5	197 (37.2)	30 (15.20	0.69 (0.42-1.14)	0.77 (0.47-1.27)	7 (3.6)	0.34 (0.13-0.86)	0.34 (0.13-0.86)
	Unclear	27 (5.1)	4 (14.8)	0.67 (0.25-1.79)	0.61 (0.26-1.44)	3 (11.1)	1.06 (0.31-3.57)	1.06 (0.31-3.57)

[a] Adjusted for days from last menstrual period, number of lifetime sex partners, acquisition of new sex partner in last 6 months, marital status, time since last abnormal pap smear, history of other sexually transmitted diseases in the last 6 months.

Table 3.8. Association between HPV detection and recency of any oral contraceptive use, with never users of oral contraceptives as reference group (N=530)

Characteristics	Total N N(col %)	Positive for any HPV n=87 (16.4%) Any HPV+ n(row %)	Unadjusted PR (95% CI)	Adjusted PR[a] (95% CI)	Positive for HR-HPV n=34 (6.4%) HR-HPV+ n(row %)	Unadjusted PR (95% CI)	Adjusted PR[a] (95% CI)
Use of oral contraceptives							
Time since last use							
Never user	95 (17.9)	21 (22.1)	Ref	Ref	10 (5.1)	Ref	Ref
Current user	63 (11.9)	10 (15.9)	0.72 (0.36-1.42)	0.67 (0.34-1.30)	4 (6.4)	0.60 (0.20-1.84)	0.44 (0.13-1.51)
Past user	372 (70.2)	56 (15.1)	0.68 (0.43-1.07)	0.73 (0.47-1.12)	20 (5.40	0.51 (0.25-1.06)	0.52 (0.26-1.04)

[a] Adjusted for days from last menstrual period, number of lifetime sex partners, acquisition of new sex partner in last 6 months, marital status, time since last abnormal pap smear, history of other sexually transmitted diseases in the last 6 months.

[65.]

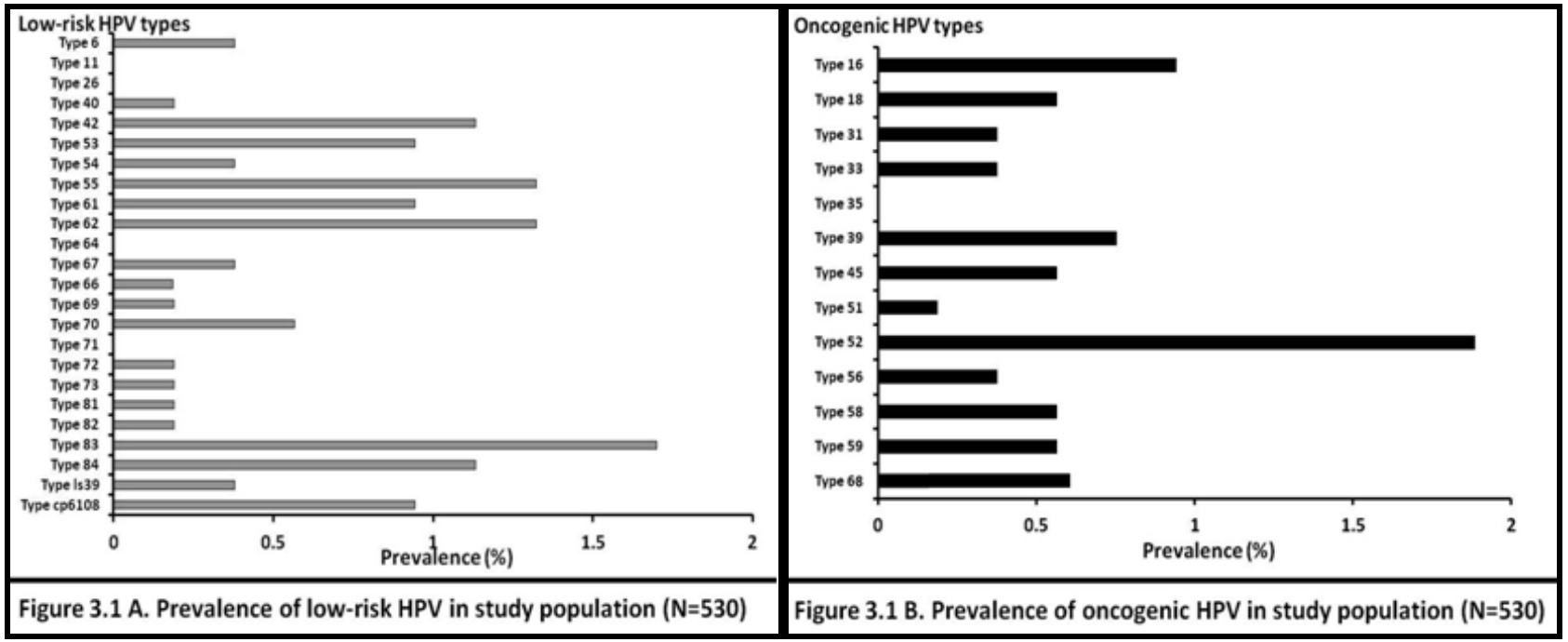

Figure 3.1. Baseline prevalence of (A) low-risk and (B) oncogenic HPV detection in study population (N=530)

CHAPTER 4:

Association of Hormonal Contraceptive Use with Cervical HPV Incidence in Pre- and Peri-menopausal Women in the U.S.

by:

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ABSTRACT

Background:

Long-term and recent use of combined oral contraceptives was suggested to associate with increased risk of cervical cancer. Study findings on the association between hormonal contraceptive use and HPV infection have been inconsistent. Most published data were drawn from women aged below 35 years. Little is known about the effect of hormonal contraceptive use on HPV detection in older women.

Objective:

The aim of this study was to assess the association of hormonal contraceptive use with new detection of HPV among pre- and perimenopausal women aged over 35 years who had normal cervical cytology. Secondary analysis was also done to compare the proportions of positive HPV detections which had persistent detection at next follow-up.

Methods:

Five hundred and thirty pre- and perimenopausal women (aged 35-54 years) were recruited from gynecologic clinics in Baltimore, MD. Subjects were followed semi-annually. Assessment was made on cervical HPV genotypes, cervical cytology, sexual behavior, demographic factors and use of hormonal contraceptives. Persistent HPV detection was defined as repeated detection of HPV at next follow-up visit as compared with previous visit. Incident detection was defined as any type-specific HPV which was detected at current visit but not at previous visit. Association between hormonal contraceptive use and incident HPV detection was measured by odds ratios (ORs) with 95% confidence intervals (CIs) estimated in generalized estimating equation models. Differences in proportions of persistent HPV detection were assessed by Chi-squared test.

Results:

Median follow-up time was 19.1 months with an interquartile range of 7.3 months. Seventy new cases of HPV and twenty-nine incident cases of high risk (HR)-HPV were observed. Relative to never users of progestin-only contraceptives (POCs), current use of POCs were found to associate with new HR-HPV detection [adjusted odds ratio (aOR): 3.24 (95% CI: 1.37-7.65) after controlling for days of menstrual cycle, number of lifetime sex partners, acquisition of new sex partner in last 6 months, marital status and time since last abnormal pap smear. No statistically significant association was detected between incident HPV detection and current use of oral contraceptives (OCs)[aOR: 0.57 (95% CI: 0.20-1.64)]. There was no significant association observed between duration of use of overall hormonal contraceptives and OCs. Association between incident HPV detection and duration of use of POCs was not assessed due to power limitations.

Among 179 positive HPV cases detected in this analysis, 61% remained HPV-positive at next follow-up visit. No significant difference in proportions with persistent infection was seen by duration or recency of hormonal contraceptive use.

Conclusions:

Our study suggested that current use of POCs was potentially associated with increased risk of incident HPV detection in short-term follow-up among pre- and perimenopausal women over the age of 35 years. Longer follow-up may be needed to better assess the longitudinal relationship between hormonal contraceptive use and HPV infection.

INTRODUCTION

Persistent infections of oncogenic HPV genotypes are believed to be a necessary but not sufficient cause of cervical cancer (1, 2). Aging (3, 4, 5) and alterations of host hormonal profile (6, 7, 8) have been implicated to play important roles in host immunity against viral infections. While long-term use of hormonal contraceptives has been demonstrated to increase risk of cervical cancer and persistent HPV infection (9, 10), most of the data were based on young female population. Few studies have looked at the effects of hormonal contraceptives on the early natural history of HPV in perimenopausal women, whose sexual behavioral and endogenous hormonal profiles are likely to be different than those of younger women. It is known that HPV prevalence peaks in the first few years after sexual debut and then declines afterwards. A second peak in prevalence has been observed at around the age of menopause in several populations in 5 world regions (2, 11). It is unclear what factors influence HPV detection in older women that might explain this second peak of HPV prevalence.

Data on determinants of HPV at older ages have been limited. Castle et al conducted a prospective study among women aged from 18 to over 65 years old in Guancaste, Costa Rica and noted that acquisition of HPV types decreased significantly as women aged, with the highest peak observed in young women and a secondary minor peak seen in older women (3). Gonzalez et al evaluated in a nested case-control study the sexual behavior and cellular immune response of women aged 45-75 years to HPV 16 virus-like particles (VLP), and reported that weaker immune response increased risk of HPV detection (4). García-Piñeres et al found that the lymphoproliferative response of

peripheral blood mononuclear cells (PBMCs) to HPV LI virus-like particles (VLPs) decreased in association with HPV persistence as well as increased age (age ≥65 years), with new HPV detection observed during follow-up among those who reported no sexual activity prior to follow-up screening (5).

The second peak in HPV prevalence observed at older ages in several populations has been postulated to arise via a number of pathways, including increased incidence due to new infections, longer persistence of HPV infections, increased detectability of infections due to cervical/vaginal changes associated with aging, and reactivation of latent infections associated with age-related immunologic and/or hormonal changes (4, 13, 14-17). Trends of HPV prevalence in succeeding birth cohorts were also hypothesized to be a contributing factor (4, 12, 13).

Our previous data showed that long-term use of progestin-only contraceptives (POCs) was associated with 3-fold increased prevalence of any HPV in women older than 35 years in the U.S. In this study, we aimed to assess with short-term prospective follow-up data the association of hormonal contraceptive use with new detection of HPV among pre- and perimenopausal women aged over 35 years who had normal cervical cytology. Secondary analysis was also done to compare the proportions of positive HPV detections by hormonal contraceptive use which had persistent detection at next follow-up visit.

MATERIALS AND METHODS

Study design

This was an observational prospective study assessing the natural history of HPV during the perimenopausal transition. Women aged 35 to 60 years were recruited from gynecological clinics associated with the Johns Hopkins Medical Institutions in Baltimore, Maryland. Exclusion criteria for the parent study included the following: (i) history of hysterectomy, organ transplant or HIV infection, (ii) currently pregnant, (iii) unable to provide informed consent, (iv) unwilling to provide contact information, (v) non-English speaking. The study protocols were reviewed and approved by the Institutional Review Board at Johns Hopkins Bloomberg School of Public Health, Baltimore, MD.

Women were followed every 6 months. Standardized questionnaires were administered by trained interviewers at baseline and follow-up visits. Information was collected on (i) use of hormonal contraceptives including duration of use, time since last use, indications of use and brand names, (ii) demographic information, (iii) sexual behavioral characteristics, (iv) reproductive history, (v) cigarette and alcohol use, (vi) use of medications other than hormonal contraceptives, (vii) history of sexually transmitted infections.

Physical examination and specimen collection

A comprehensive physical examination, as well as pelvic examination if indicated, was performed at baseline visit. A cervical secretion specimen, liquid-based Pap smear and cervical swab (Digene HPV sampler kit) were obtained. Cervical secretions were collected using a Merocel ophthalmic sponge which was placed at 6 o'clock of the

cervical os for 30 seconds. The collected cervical sample was stored at -80 ^{o}C until testing.

Liquid-based Pap smears were performed where clinically indicated. Results of pap smears were abstracted from medical records and the cytological abnormalities were classified based on the 2001 Bethesda classification scheme [normal, inflammation, ASCUS (aytpical squamous cells – undetermined significance), LSIL (low grade squamous intraepithelial lesion), HSIL (high grade squamous intraepitheilial lesion)].

HPV DNA type-specific testing was done on cervical swab samples. DNA was extracted using the QIAamp DNA Blood Kit (Qiagen, Courtaboeuf, France) according to manufacturer's instructions with modification. After extraction, 8 µls of DNA (4% of total volume of extracted DNA) was amplified using the PGMY09/11 L1 consensus primer system, which amplifies with high efficiency over 40 HPV genotypes known to infect the genital tract. Genotype discrimination will be performed by hybridizing 40 µl of the PCR product to a membrane with immobilized probes targeting 37 HPV genotypes (HPV Linear Array Roche Molecular Systems, Roche Diagnostics, Indianapolis, IN).

Measures and definition of terms

Primary exposure of interest

Visits were assessed in consecutive pairs. The primary exposure variables being measured were current use of (i) any hormonal contraceptives, (ii) progestin-only contraceptives (POCs), and (iii) oral contraceptives (OCs). The category of OCs

included participants reporting the use of "combined oral contraceptives (COC)", medication brand names that can be classified pharmaceutically as COC , as well as the "pill(s)," which are usually referred to as COCs based on generally accepted notion (18, 19). Duration of use of any hormonal contraceptives and OCs were assessed in secondary analyses.

Outcome of interest

Primary outcome variables were evaluated against the exposure variables: (i) new detection of any HPV type in cervical sample which was not found at previous visit; (ii) new detection of any 1 of the 13 oncogenic HPV types in cervical samples which was not found at previous visit. Hence participants would be at risk at every visit for new HPV-type-specific infection that was not detected in previous visit. Oncogenic HPV types were defined as HPV types16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 as proposed by the International Agency for Research on Cancer (20).

Secondary outcome of interest was repeated detection of any HPV type that was found at previous visit. Positive HPV cases included both prevalent baseline detection of HPV and new detection of HPV during follow-up.

Other measures

All variables were coded categorically. For incidence and persistence analyses, the following characteristics were coded as time-varying factors: (i) primary exposure variables including current use of any hormonal contraceptives, POCs and OCs, (ii) sexual activity within 6 months prior to visit, (iii) days since last menstrual period, (iv)

history of other sexually transmitted infections within 6 months prior to visit, (v) marital status, (vi) smoking status. Others factors assessed were treated as time-fixed variables as defined at baseline, which included demographic characteristics such as age, race, parity, lifetime number of sexual partners, history of colposcopy and time since last abnormal Pap smear.

Statistical analysis

Analysis population

A total of 1244 visit pairs were observed during follow-up. Population or visits eligible for analysis included those who (i) had at least 6-month follow-up (n=383, 72.3% of population at baseline), (ii) remained cytologically normal during follow-up (n=519, 97.9%), and (iii) visits that were associated with pre- or perimenopausal status (n=1057, 85%). The following visits were excluded from the visit-pair analyses: (i) visits with missing menopausal status (n=23, 1.9%), (ii) visits with missing recent sexual behavioral information (n=101, 8.1%)

Univariable and multivariable modeling

Generalized estimating equation (GEE) models were used to obtain the odds ratios with 95% confidence intervals for incident HPV detection during follow-up for the average subject population. Exchangeable correlation was fit with the assumption of same correlation between any pair of observations. Covariates which yielded statistically significant association or potential statistically significant association ($p<0.10$) in univariable models were considered in the final multivariable model to obtain the

adjusted odds ratio for the association between the primary exposure and outcome event of interest.

Selection of predictors was done by forward and backward selection of factors with cut-off P value at 0.05. Participants' demographic characteristics, baseline reproductive health history, sexual behavioral factors, and primary partner characteristics were assessed as potential confounding factors. Variables were defined as confounders if the odds ratio for the primary exposure variable changed by ≥10% when the variable was added to the primary model. These confounders were retained in final multivariable models. If supported by literature to be important risk factors for HPV progression, predictors which did not yield statistically significant association were also included in the final model based on epidemiologic considerations even though the final model was not the most parsimonious. Models by default included age at baseline. Interaction terms were used to assess effect modification by each of the variables included in the final multivariable model.

Sensitivity analysis

Sensitivity analyses were performed with (i) inclusion or exclusion of visit pairs that were more than 8 months apart., (ii) inclusion or exclusion of categories of days between visits in multivariable model.

All analyses were done using Stata/SE version 11 (StataCorp, College Station, TX).

RESULTS

Study population

Five-hundred and thirty pre- and perimenopausal women were followed for HPV detection. Three hundred and eighty-three (72.3%) had at least 6-month follow-up at the time of analysis (Appendix. Appendix Table 4.1). The population that was included in analysis was not significantly different from the population at enrollment with regard to baseline hormonal contraceptive use, demographic characteristics, sexual behavioral factors, history of abnormal Pap smears and other sexually transmitted infection (Appendix. Appendix Table 4.1). The analysis population contributed a total of 928 visit pairs (Table 4.1), with a median follow-up time of 19.1 months (interquartile range (IQR) of 7.3 months). The median time between consecutive visits was 6.4 months (IQR: 2.4 months). Eighty-three (8.9%) visit pairs were more than 1 year apart. Two-hundred and six visit-pairs were more than 8 months apart (25.8%).

Analysis of incident HPV detection

A total of 41 women had new HPV types detected. Seventy new cases of any HPV and twenty-nine incident cases of HR-HPV were detected during follow-up. The most common incident HR-HPV types were HPV type 16 (n=5, 17.2%) and 58 (n=4, 13.8%) (Figure 4.1). Women having new detection of HPV were more likely to be single ($P<0.001$), having sex with new partners in the past 6 months ($P<0.001$), or having more than 5 lifetime sex partners at baseline ($P<0.001$)(Table 4.1). These characteristics remained independent risk factors for HPV incident detection in multivariable analysis after controlling for other confounding factors and hormonal contraceptive use (Table 4.3).

Women who reported current use of POCs were more likely to have incident HR-HPV (P=0.02)(Table 4.2). A greater proportion of current POC users had new detection of any HPV (11.4%) than never POC users (7.4%) though the estimate did not reach statistical significance (Table 4.2). In multivariable analysis, current use of POCs was found to associate with 3-fold increased risk of incident HR-HPV [adjusted odds ratio (aOR): 3.24 (95% CI: 1.37-7.65)] after controlling for last menstrual period, acquisition of new sex partner since last visit, marital status since last visit, history of other sexually transmitted diseases since last visit, time between visits, number of lifetime sex partners at baseline, time since last abnormal pap smear at baseline (Table 4.4). Relative to never users, current use of POCs was also associated with higher odds of new detection of any HPV with borderline statistical significance [aOR: 1.64 (95% CI: 0.85-3.19)] (Table 4.4). Past use of POCs was not noted in our analysis to associate with incident detection of any HPV or HR-HPV. No significant association was seen between new detection of HPV and recency or duration of use of any hormonal contraceptives or OCs (Tables 4.5-4.7). Similar observations were observed with inclusion or exclusion of visit pairs that were more than 8 months apart in the analysis population as well as inclusion and exclusion of categories of days between screening visits in the multivariable model (results not shown).

Secondary analysis of persistent HPV detection

A secondary analysis of persistent HPV infection at 6-month follow-up was also done. Among women who had at least 6-month follow-up (n=383, 72.3%), 110 (61%) of HPV cases had persistent detection at the next follow-up visit (Appendix. Appendix Table 4.2). No significant association was observed between persistent detection of HPV and the

recency or duration of use of hormonal contraceptives, POCs or OCs, or other population characteristics (Appendix. Appendix Table 4.3). The results remained robust when visit pairs that were more than 8 months apart were excluded from the analysis population (results not shown).

DISCUSSION

We have previously reported that more than 5 years' use of POCs was associated with 3-fold increased prevalence of any HPV, and current use of POCs was found to associate with increased HPV prevalence with the estimate bordering on statistical significance. HPV prevalence is mediated by both the incidence and persistence of infection. Our analyses on short-term prospective data revealed that current POC use was potentially linked to increased incident HPV detection. No significant positive association was detected between oral contraceptive (OC) use and HPV prevalent or incident detection in our study population.

The majority of OCs belong to the category of COCs (18, 19). Association between COC and HPV prevalence was noted in younger female populations (10, 21-27). It has been proposed that the association between COC and HPV infection is mainly driven by COC's effect on HPV persistence and not on acquisition (28). A previous study reported that prevalent carcinogenic HPV infections persisted more often in women aged ≥ 42 years than in younger women, and persistent prevalent infections were associated with most of the CIN 2 or CIN3 lesions diagnosed in those females in that study (29). In our analysis on short-term follow-up, no significant difference in proportions of persistent prevalent/incident HPV detection at 6 months was seen in association with overall

hormonal contraceptive use. Perimenopausal women in our study were recruited from those who were routinely screened with Pap smears at gynecologic clinics and had been cytologically normal at baseline and during follow-up. It is possible that, should long-term COC or other hormonal contraceptive use lead to persistent HPV infection which progresses into CIN lesion, these lesions would have been detected and treated in women during screening prior to study entry, hence resulting in the apparent lack of OC-associated persistent prevalent HPV detection seen in our study. It is worthy to note that likelihood of persistence among newly detected HPV cases in women has not been shown to increase by age (29). This finding suggests that, in older women, increased HPV prevalence is not likely to be explained by increased persistence of new HPV detection.

A positive association was observed between current POC use and HPV incident detection in our population. POCs, particularly DMPA, has been reported in other epidemiologic studies to associate with HIV acquisition in women (30, 32), although the finding has not been consistently reported across studies due to different study designs and population characteristics (33, 34).

Progestin has been postulated to promote viral detection by causing cervical or vaginal changes that increase viral detectability. Natural progesterone during the luteal phase of menstrual cycle was found to reduce vaginal thickness (35, 36). High concentrations of progesterone in the luteal phase were linked to increased susceptibility to simian immunodeficiency viral infection in Pigtail macaques (37).The effect of synthetic progestin, e.g. DMPA, on vaginal epithelium has also been investigated in human cohorts.

Miller et al observed that short-term (6 months) use of DMPA led to lowered serum estradiol (35) as seen in other studies (38, 39), as well as changes in the vagina including a statistical significant though small decrease in cell layers associated with reduction of H_2O_2-positive *Lactobacillus* and vaginal glycogen. Others failed to detect such vaginal thinning effect with 1 single injection of DMPA (38), or with long-term (2-3 years) of DMPA use in case-control study (39). These studies, however, were done in premenopausal women, and data on the effect of DMPA or POC on endocervical or vaginal epithelium in perimenopausal women are lacking. Atrophic vaginitis is a common ailment in late perimenopausal and early menopausal women. Perimenopause is characterized by a mix of ovulatory and anovulatory cycles accompanied by irregularities of endogenous estrogen (40). The hypoestrogenic effect of progesterone is likely augmented when endogenous estrogen drops to low levels, thereby creating a "window of vulnerability" in which short-term vaginal changes can occur, which in turn may perturb the epithelial barrier to HPV infection, and facilitate seeding/shedding of viral particles at/from the basal layer of the vaginal epithelium. In contrast to DMPA, COC use had not been found to affect the gross, colposcopic, or histologic appearance of the vaginal epithelium or characteristics of vaginal or cervical discharge (41).

Sex hormones have been shown to influence immune responses in the female genital tract (42-45). Hormonal changes or imbalance can suppress host immune responses which in turn may induce reactivation of a latent virus. Alternatively, immunosuppression can impair clearing of a newly acquired or reactivated infection between screening visits, resulting in an "incident" detection. Progesterone has been suggested by experimental

studies to have a general suppressive effect of on mucosal immunity. *In vitro* studies showed that progesterone, at pregnancy concentrations, can lead to increased production of IL4 and IL5 and reduced regulatory T cell function (46, 47). Exposure of HPV16 VLP-stimulated PBMCs to estradiol and progesterone, either alone or in combination, was shown to significantly decrease the levels of lymphoproliferation and production of proinflammatory cytokines (48). In mouse models of herpes simplex 2 (HSV2) infection, DMPA administration led to significant lowering of local HSV2-specific immunoglobulin G (IgG) and IgA in their vaginal washes (49), resulting in dramatic increased susceptibility to HSV2 infection. Given the immunosuppressive effects of POC, it is reasonable to infer that POC may impair clearing of human HPV detection. A previous study on younger women reported a mildly positive association (OR51.15; 95% CI: 1.01–1.32) between current use of injectable contraceptive and persistent HPV detection among those with mild or abnormal cytology (50), although the association was not consistently reported in others (51).

Our study was an analysis of short-term follow-up data and was therefore limited in power to study the association between duration of POC use and HPV incidence or persistence. In addition, we were limited on self-report for information on sexual risk behaviors, making the observed associations subject to potential unmeasured and residual confounding. Our study population reported low level of new sex exposure prior to baseline (2.8%) and during follow-up (3.3%). The confounding effect of unmeasured sexual behavior on the associations assessed would therefore likely be small when compared to studies done in younger female population.

In summary, our analyses showed that current use of POCs was potentially associated with increased risk of incident HPV detection in short-term follow-up among women over the age of 35 years. Such association had not been reported between HPV incidence and COC or POC in younger female population (28, 51-56). This contrast suggests that the nature of incident HPV detection in younger and older females may not be the same. Incident HPV detection can theoretically represent either a newly acquired infection, or detection of a previously acquired infection that has "re-emerged" from latency (13). In a sexually active younger female population, more of the incident HPV detections are likely newly acquired infections. Older women, however, tend to have more stable sexual relationships (57), and incident detection may possibly represent a mixed pool of cases with higher proportion of viral reactivation instead of just newly acquired infection. Long-term follow-up data focused on older women will allow us to better assess the longitudinal relationship between HC use and HPV incidence and persistence. Further studies are also warranted to evaluate the biologic impact of POC and other hormonal contraceptives on the human genital immune milieu in older women in relation to HPV infection.

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TABLES AND FIGURE

Table 4.1. Characteristics of study population and incident HPV detection during follow-up (Total number of visit pairs: 928)

Characteristics at follow-up visit	Total visit pairs N=928 (col %)[a]	Incident detection of any HPV since last visit n=70 (7.5%) (row %)	P	Incident detection of any HR-HPV since last visit n=29 (3.1%)[b] (row %)	P
Demographic characteristics					
Age at enrollment years)			0.75		0.85
35-39	244 (26.3)	16 (6.6)		8 (3.3)	
40-44	249 (26.8)	20 (8.0)		9 (.3.6)	
45-49	305 (32.9)	26 (8.5)		10 (3.3)	
≥50	123 (13.3)	8 (6.2)		2 (1.6)	
Race at enrollment			0.34		0.75
Caucasian	713 (76.8)	52 (7.3)		23 (3.2)	
African American	150 (16.2)	15 (10.0)		5 (3.33)	
Others[c]	65 (7.0)	3 (4.6)		1 (1.5)	
Marital status since last visit			**<0.001**		**0.01**
Married	616 (66.4)	26 (4.2)		13 (2.1)	
Single	169 (18.2)	19 (11.2)		6 (3.6)	
Divorced/separated/widowed	143 (15.4)	25 (17.5)		10 (7.0)	
Smoked since last visit			0.12		0.63
No	854 (92.0)	61 (7.1)		26 (3.0)	
Yes	74 (8.0)	9 (12.2)		3 (4.1)	
Parity at enrollment			0.19		0.22
0 or 1	302 (32.6)	27 (8.9)		11 (3.6)	
2	197 (21.3)	18 (9.1)		9 (4.6)	
>2	427 (46.1)	25 (5.9)		9 (2.1)	
Sexual behavioral characteristics					
Frequency of sex since last visit (times/month)			0.09		0.29
None	72 (7.8)	8 (11.1)		3 (4.2)	
1-2	163 (17.6)	14 (8.6)		6 (3.7)	
3-4	198 (21.3)	7 (3.5)		2 (1.0)	
≥5	495 (53.3)	41 (8.3)		18 (3.6)	
Sexual activity since last visit			**<0.001**		0.10

No sex	150 (16.2)	12 (8.0)		5 (3.3)	
Have sex but no new sexual partner	747 (80.5)	49 (6.6)		21 (2.8)	
Have sex with new sexual partner	31 (3.3)	9 (29.0)		3 (9.7)	
Lifetime number of sexual partners			**<0.001**		**0.001**
<5	375 (40.4)	9 (2.4)		2 (0.5)	
5-10	352 (37.9)	40 (11.4)		18 (5.1)	
>10	201 (21.7)	21 (10.5)		9 (4.5)	
Last menstrual period (days)			0.67		0.25
1-14	410 (44.2)	34 (8.3)		15 (3.7)	
15-28	332 (35.8)	23 (6.9)		8 (2.4)	
28-40	62 (6.7)	6 (9.7)		4 (6.5)	
40-365	124 (13.4)	7 (5.7)		2 (1.6)	
Ever had colposcopy			0.56		0.21
No	732 (78.9)	53 (7.3)		20 (2.8)	
Yes	197 (21.2)	17 (8.5)		9 (4.5)	
Ever had treatment for abnormal pap smear			0.34		0.31
No	768 (82.8)	55 (7.2)		22 (2.9)	
Yes	160 (17.2)	15 (9.4)		7 (4.4)	
Time since last abnormal pap smear			0.05		**0.03**
Never had abnormal pap smear	521 (56.1)	30 (5.8)		9 (1.7)	
<1 year ago	29 (3.1)	3 (10.3)		1 (3.5)	
2-5 years ago	89 (9.6)	12 (13.5)		6 (6.7)	
≥6 years ago	289 (31.1)	25 (8.7)		13 (4.5)	
Diagnosed with other sexually transmitted infections in the past 6 months [d]			0.62		0.06
No	909 (98.0)	68 (7,.5)		27 (3.0)	
Yes	19 (2.1)	2 (10.5)		2 (10.5)	

[a.] Percentages or numbers in categories may not add up to 100% or the total number due to missing data.
[b.] HR-HPV types: oncogenic HPV as defined as types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 (IARC 2007).
[c.] Other categories include American Indian (n=2), Pacific Islander (n=2), Asian (n=16), unidentified (n=9).
[d.] Other sexually transmitted infections included genital chlamydia, gonorrhea, syphilis, herpes, trichomoniasis, chancroid, warts.

Table 4.2. Hormonal contraceptive use and new HPV detection in study population (Total number of visit pairs: 928)

Characteristics at current follow-up visit		Total visit pairs N=928 (col %)	Incident detection of any HPV since last visit n=70 (7.5%) (row %)	P	Incident detection of any HR-HPV since last visit N=29 (3.1%) (row %)	P
Use of any hormonal contraceptives						
				0.77		0.93
Duration of use	**Years**					
Never used any HC		91 (9.8)	5 (5.5)		2 (2.2)	
Current	≤5	365 (39.3)	26 (7.1)		13 (3.6)	
	>5	41 (4.4)	5 (12.2)		1 (2.4	
Former	≤5	151 (16.3)	10 (6.6)		3 (2.0)	
	>5	253 (27.3)	22 (8.7)		9 (3.6)	
Unclear duration		27 (2.9)	2 (7.4)		1 (3.7)	
Time since last use at last visit				0.38		0.73
Never user		91 (9.8)	5 (5.5)		2 (2.2)	
Current user		202 (21.8)	18 (8.9)		8 (4.0)	
Last use ≤ 5 years ago		194 (20.9)	13 (6.7)		8 (4.1)	
Last use 6-10 years ago		104 (11.2)	4 (3.9)		3 (2.9)	
Last use >10 years ago		337 (36.3)	30 (8.9)		8 (2.4)	
Use of any progestin-only contraceptives						
Duration of use	**Years**			0.27		0.18
Never used any progestin-only contraceptives		691 (74.5)	48 (7.0)		17 (2.5)	
Ever users	≤5	208 (22.4)	19 (9.1)		10 (4.8)	
	>5	26 (2.8)	2 (7.7)		2 (7.7)	
Cannot be determined		3 (0.3)	1 (33.3)		0 (0)	
Time since last use at last visit				0.27		**0.02**
Never used any progestin-only contraceptives		691 (74.5)	48 (7.0)		17 (2.5)	
Current user		105 (11.3)	12 (11.4)		8 (7.6)	
Past user		132 (14.2)	10 (7.6)		4 (3.0)	

Use of oral contraceptives						
Duration of use	**Years**			0.34		0.86
Never used any oral contraceptives		164 (17.7)	15 (9.2)		4 (2.4)	
Ever users	≤5	380 (41.0)	23 (6.1)		11 (2.9)	
	>5	338 (36.4)	30 (8.9)		12 (3.6)	
Cannot be determined		46 (5.0)	2 (4.4)		2 (4.4)	
Time since last use				0.66		0.13
Never used any oral contraceptives		164 (17.7)	15 (9.2)		4 (2.4)	
Current user		93 (10.2)	6 (6.5)		0 (0)	
Past user		671 (72.3)	49 (7.3)		25 (3.7)	

Table 4.3. Association between any hormonal contraceptive use and new HPV detection during follow-up (Total number of visit pairs: 928)

Characteristics at current follow-up visit	Total visit pairs N=928	Incident detection of any HPV n=70 (7.5%)			Incident detection of HR-HPV n=29 (3.1%)		
	N(col %)	Incident detection n(row %)	Unadjusted OR (95% CI)	Adjusted OR[a] (95% CI)	Incident detection n(row %)	Unadjusted OR (95% CI)	Adjusted OR[a] (95% CI)
Use of any hormonal contraceptives							
Last time of use							
Never user	91 (9.8)	5 (5.5)	Ref	Ref	2 (2.2)	Ref	Ref
Current user	202 (21.8)	18 (8.9)	1.81 (0.68-4.79)	1.23 (0.39-3.86)	8 (4.0)	1.88 (0.40-8.76)	0.97 (0.18-5.06)
Past user	635 (68.4)	47 (7.4)	1.47 (0.59-3.67)	1.05 (0.35-3.15)	19 (3.0)	1.44 (0.34-6.14)	0.76 (0.15-3.94)
Time from last menstrual period							
Days							
1-14	410 (44.2)	34 (8.3)	Ref	Ref	15 (3.7)	Ref	Ref
15-28	332 (35.8)	23 (6.9)	0.83 (0.47-1.46)	1.17 (0.63-2.18)	8 (2.4)	0.66 (0.26-1.70)	1.20 (0.46-3.08)
28-40	62 (6.7)	6 (9.7)	0.60 (0.24-1.53)	0.40 (0.09-1.78)	4 (6.5)	1.92 90.64-5.76)	0.48 (0.06-3.94)
40-365	124 (13.4)	7 (5.7)	1.24 (0.52-2.99)	1.32 (0.60-2.92)	2 (1.6)	0.43 (0.10-1.82)	0.80 (0.23-2.81)
Lifetime number of sexual partners							
<5	125 (13.5)	9 (2.4)	Ref	Ref	2 (0.5)	Ref	Ref
6-10	250 (26.9)	40 (11.4)	**4.81 (2.20-10.54)**	**4.32 (2.01-9.29)**	18 (5.1)	9.39 (2.19-40.29)	**7.89 (1.86-33.50)**
>10	553 (59.6)	21 (10.5)	**4.33 (1.86-10.10)**	**3.01 (1.23-7.38)**	9 (4.5)	8.17 (1.74-38.31)	**5.38 (1.11-26.04)**
Sexual activity in last 6 months							
No sex	150 (16.2)	12 (8.0)	Ref	Ref	5 (3.3)	Ref	Ref
Have sex but no new sexual partner	747 (80.5)	49 (6.6)	0.80 (0.42-1.51)	1.45 (0.66-3.18)	21 (2.8)	0.84 (0.32-2.23)	1.07 (0.35-3.24)
Have sex with new sexual partner	31 (3.3)	9 (29.0)	**4.22 (1.55-11.49)**	**3.60 (1.25-10.36)**	3 (9.7)	3.00 (0.67-13.41)	2.70 (0.61-11.91)
Marital status							
Married	616 (66.4)	26 (4.2)	Ref	Ref	13 (2.1)	Ref	Ref
Single	169 (18.2)	19 (11.2)	**2.97 (1.55-5.74)**	**2.73 (1.28-5.84)**	6 (3.6)	1.64 (0.55-4.90)	1.29 (0.35-4.75)
Divorced/separated/widowed	143 (15.4)	25 (17.5)	**4.84 (2.60-9.00)**	**3.45 (1.72-6.90)**	10 (7.0)	**3.30 (1.37-7.90)**	2.32 (0.89-6.00)

Time since last abnormal pap smear							
Never	521 (56.1)	30 (5.8)	Ref	Ref	9 (1.7)	Ref	Ref
< 1 year ago	29 (3.1)	3 (10.3)	1.94 (0.61-6.13)	2.17 (0.73-6.44)	1 (3.5)	2.01 (0.28-14.45)	2.01 (0.35-11.65)
2-5 years ago	89 (9.6)	12 (13.5)	**2.61 (1.24-5.49)**	1.74 (0.78-3.85)	6 (6.7)	**4.13 (1.48-11.57)**	**3.56 (1.15-11.06)**
≥ 6 years ago	289 (31.1)	25 (8.7)	1.55 (0.85-2.82)	1.22 (0.66-2.26)	13 (4.5)	**2.68 (1.10-6.54)**	2.19 (0.90-5.36)

[a] Adjusted for days from last menstrual period, acquisition of new sex partner since last visit, marital status since last visit, time since last abnormal pap smear at baseline, number of lifetime sex partners at baseline.

Table 4.4. Association between new HPV detection and recency of any progestin-only hormonal contraceptive use (Total number of visit pairs: 928)

Characteristics at follow-up visit	Total visit pairs N=928	Incident detection of any HPV n=70 (7.5%)			Incident detection of HR-HPV n=29 (3.1%)		
	N(col %)	Incident detection n(row %)	Unadjusted OR (95% CI)	Adjusted OR[a] (95% CI)	Incident detection n(row %)	Unadjusted OR (95% CI)	Adjusted OR[a] (95% CI)
Use of progestin-only contraceptives							
Last time of use							
Never user	691 (74.5)	48 (7.0)	Ref	Ref	17 (2.5)	Ref	Ref
Current user	105 (11.3)	12 (11.4)	1.77 (0.94-3.34)	1.64 (0.85-3.19)	8 (7.6)	**3.22 (1.39-7.44)**	**3.24 (1.37-7.65)**
Past user	132 (14.2)	10 (7.6)	1.06 (0.49-2.28)	1.15 (0.48-2.75)	4 (3.0)	1.22 (0.41-3.62)	1.32 (0.42-4.09)

[a] Adjusted for days from last menstrual period, acquisition of new sex partner since last visit, marital status since last visit, time since last abnormal pap smear at baseline, number of lifetime sex partners at baseline.

Table 4.5. Association between new HPV detection and recency of oral contraceptive use (Total number of visit pairs: 928)

Characteristics at follow-up visit	Total visit pairs N=928	Incident detection of any HPV n=70 (7.5%)			Incident detection of HR-HPV n=29 (3.1%)		
	N(col %)	Incident detection n(row %)	Unadjusted OR (95% CI)	Adjusted OR[a] (95% CI)	Incident detection n(row %)	Unadjusted OR (95% CI)	Adjusted OR[a] (95% CI)
Use of oral contraceptives							
Last time of use							
Never user	164 (17.7)	15 (9.2)	Ref	Ref	4 (2.4)	Ref	Ref
Current user	93 (10.2)	6 (6.5)	0.64 (0.24-1.71)	0.57 (0.20-1.64)	0 (0)	na	na
Past user	671 (72.3)	49 (7.3)	0.81 (0.43-1.51)	0.63 (0.30-1.33)	25 (3.7)	na	na

[a] Adjusted for days from last menstrual period, acquisition of new sex partner since last visit, marital status since last visit, time since last abnormal pap smear at baseline, number of lifetime sex partners at baseline.
na: could not be assessed due to "0" and low counts in the categories

Table 4.6. Association between new HPV detection and duration of any hormonal contraceptive use (Total number of visit pairs: 928)

Characteristics at follow-up visit		Total visit pairs N=928	Incident detection of any HPV n=70 (7.5%)			Incident detection of HR-HPV n=29 (3.1%)		
		N(col %)	Incident detection n(row %)	Unadjusted OR (95% CI)	Adjusted OR[a] (95% CI)	Incident detection n(row %)	Unadjusted OR (95% CI)	Adjusted OR[a] (95% CI)
Use of any hormonal contraceptives								
Duration of most recent use	**Years**							
Never user		91 (9.8)	5 (5.5)	Ref	Ref	2 (2.2)	Ref	Ref
Ever user								
	≤5	516 (55.6)	36 (7.0)	1.45 (0.57-3.69)	0.92 (0.30-2.82)	16 (3.1)	1.57 (0.37-6.75)	0.76 (0.15-3.82)
	>5	294 (31.7)	27 (9.2)	1.72 (0.66-4.45)	1.49 (0.48-4.62)	10 (3.4)	1.51 (0.32-7.15)	0.91 (0.16-5.07)
Cannot be determined		27 (2.9)	2 (7.4)	1.26 (0.26-6.21)	0.96 (0.15-5.03)	1 (3.7)	1.55 (0.14-17.4)	0.88 (0.06-13.83)

[a] Adjusted for days from last menstrual period, acquisition of new sex partner since last visit, marital status since last visit, time since last abnormal pap smear at baseline, number of lifetime sex partners at baseline.

58.

Table 4.7. Association between new HPV detection and duration of oral contraceptive use (Total number of visit pairs: 928)

Characteristics at follow-up visit		Total visit pairs N=928	Incident detection of any HPV n=70 (7.5%)			Incident detection of HR-HPV n=29 (3.1%)		
		N(col %)	Incident detection n(row %)	Unadjusted OR (95% CI)	Adjusted OR[a] (95% CI)	Incident detection n(row %)	Unadjusted OR (95% CI)	Adjusted OR[a] (95% CI)
Use of oral contraceptives								
Duration of most recent use	**Years**							
Never user		164 (17.7)	15 (9.2)	Ref	Ref	4 (2.4)	Ref	Ref
Ever user								
	≤5	380 (41.0)	23 (6.1)	0.66 (0.32-1.35)	0.47 (0.20-1.08)	11 (2.9)	1.22 (0.39-3.75)	0.78 (0.24-2.48)
	>5	338 (36.4)	30 (8.9)	0.97 (0.50-1.89)	0.89 (0.40-1.98)	12 (3.6)	1.45 (0.46-4.61)	1.10 (0.32-3.73)
Cannot be determined		46 (5.0)	2 (4.4)	0.45 (0.11-1.93)	0.36 (0.08-1.65)	2 (4.4)	1.78 (0.34-9.26)	1.56 (0.26-9.22)

[a] Adjusted for days from last menstrual period, acquisition of new sex partner since last visit, marital status since last visit, time since last abnormal pap smear at baseline, number of lifetime sex partners at baseline.

59.

Figure

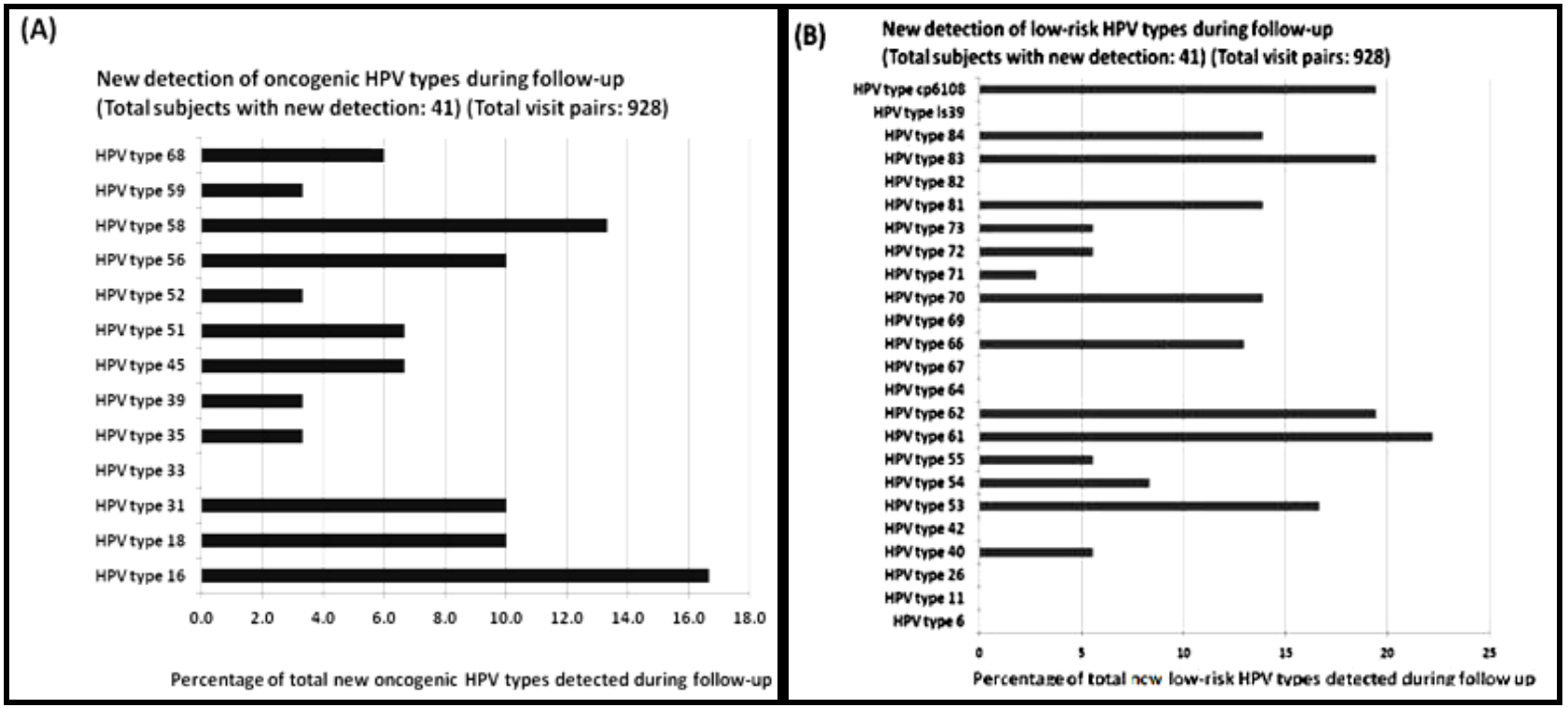

Figure 4.1. New detection of (A) oncogenic and (B) low-risk HPV types during follow-up (total visit pairs: 928)

CHAPTER 5:

Association of Hormonal Contraceptive Use with Cervical Cytokine Milieu in Pre- and Perimenopausal Women in the U.S.

by

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ABSTRACT

Background:

Long-term and recent use of combined oral contraceptives was suggested to associate with increased risk of cervical cancer. Study findings on the association between hormonal contraceptive use and HPV infection have been inconsistent. Most data were drawn from women aged below 35 years. Little is known about the effect of hormonal contraceptive use on host cervical immune milieu and HPV detection in older women.

Objective:

The aim of this study was to compare the host cervical cytokine profiles between current and non-current hormonal contraceptive users among HPV-negative women who were aged over 35 years.

Methods:

Pre- and perimenopausal women (aged 35-54 years) were recruited from gynecologic clinics in Baltimore, Maryland. Pairs of cervical secretion samples were collected from 105 current hormonal contraceptive users and 105 non-current users who were matched on 5-year age groups. Subjects tested were (i) HPV-negative at baseline, (ii) did not have other recent genital infections or abnormal Pap smears, (iii) did not report use exogenous sex hormones other than hormonal contraceptives in the past 6 months. Extracted specimens from cervical secretion samples were tested by a polystyrene bead-based multiplex assay for 27 immune markers (cytokines, chemokines and growth factors; BioRad Human Group 1 panel). Cytokine measurements were normalized to total

milligrams of protein to correct for sampling variability. Comparison of individual mean cytokine levels were made using paired-t-test between user groups of hormonal contraceptives. Collective comparison was done using Spearman's rank correlation coefficients to determine correlations for all pairwise combinations of the 27 cytokines, chemokines, and growth factors measured. Differences between correlation coefficients were tested by z-test of the Fisher's r to z-transformation of the Spearman rho correlation coefficients.

Results:

Cytokine correlation patterns differed between current and non-current hormonal contraceptive users. In non-current users, there were significantly more positive correlations detected among proinflammatory cytokines, most notably IP10, MCP1, MIP1a and RANTES, as well as between proinflammatory and immunoregulatory cytokines. Overall cytokine correlations were weaker among current hormonal contraceptive users than non-current users.

Relative to non-current hormonal contraceptive users, current progestin-only contraceptive (POC) users were found to have statistically significantly lower levels of IP10, IL-12, IL-13, IL-15, while current combined oral contraceptive (COC) users had significantly lower levels of IL-2, IP10 and MCP1. Compared to COC users, POC users appeared to show fewer significant correlations among immunoregulatory cytokines, as well as a suggestion of a more predominant Th2-skewed profile as evidenced by significant correlations between IL-4, IL-10, and IL-13. The findings suggested differential impact of different types of exogenous hormones on cervical cytokine milieu.

Conclusions:

Our analyses revealed differences in cytokine profiles between current and non-current hormonal contraceptive users in HPV-negative women. Correlation analyses of cytokine measurements hinted at effects of hormonal contraceptive use on coordinated immune response in the cervix, which in turn may potentially affect host responses to local genital infections.

INTRODUCTION

Cervical cancer is the third most common gynecologic cancer in women in the U.S. (1). Uncontrolled persistent infection with a high-risk type human papillomavirus (HPV) has been postulated to be an essential but not sufficient cause for cervical dysplasia and neoplasia (2). The risk of HPV persistence is significantly higher among immunosuppressed patients (3-6). Other host exposure factors, such as use of hormonal contraceptives and smoking, were observed to increase the risk of persistent HPV infection and cervical cancer (7-10), suggesting that interaction of viral factors, local immunity and hormonal status underlies the detection, progression and carcinogenesis of HPV infection (11).

Both the innate and cellular immune systems are important determinants for clearance of cervical HPV infection (12-14). Epithelial cells lining the cervicovaginal mucosa are able to express cytokines, sex hormone receptors and genital tract-specific defensins (15-16), and hence are vital components in mucosal innate and adaptive immunity (17). Cytokines are constitutively expressed by the cervicovaginal cells at low levels in a para- and autocrine fashion (18), and are also secreted by immune effector cells during immune activation. Cytokines can generally be classified into a number of categories according to the nature of their actions on the immune system (19, 20): (i) "proinflammatory" cytokines that contribute to the inflammatory process by activating leucocytes, e.g. IL-1, TNF-α, RANTES, (ii) Immunoregulatory/proinflammatory cytokines that stimulate inflammation and cell-mediated responses (Th1), e.g. IL-2, IL-12, IFN-γ, (iii) Immunoregulatory/anti-inflammatory cytokines that mediate humoral immunity as well as allergic and parasitic immunity (Th2), e.g. IL-4, IL-5, IL-9, IL-10, IL-13, (iv) Th17-

associated cytokines, such as IL-17 and IL-22 (18), (v) growth factors, such as GCSF, GMCSF, VEGF (19-20). A local Th1 response has been observed in women who cleared HPV infection (21), while Th2 response cytokines were found to be increased in HIV-positive HPV-co-infected persons compared to those infected with HPV alone or not infected (22-24). These findings indicated that a shift from Th1 to Th2 response might contribute to HPV persistence.

A number of past studies on immunity to HPV were limited in using serum cytokine levels as surrogate markers for cervical immunity (reviewed in 25), while in reality serum and cervical cytokine concentrations are not well correlated (12). This lack of correlation was observed similarly in women with recent HPV infection, hinting that HPV infection may not cause gross changes in epithelial permeability that result in serum transudation of cytokines (12). Factors influencing local mucosal immunity against viral infection are still not fully elucidated. Use of oral contraceptives has been shown in observational studies to correlate with elevated cervical IL-10 and IL-12 levels (26, 27). A previous study showed that treatment of peripheral blood mononuclear cells *in vitro* with biologically relevant concentrations of estrogen and progesterone led to suppressed expression of proinflammatory cytokines and increased expression of anti-inflammatory cytokines in response to HPV16 virus-like particle exposure (28). Most of the previous studies on cervical immune milieu, however, are focused on young women, and baseline data in older women have been lacking.

We have previously shown that long-term use of progestin-only contraceptives (POCs) is associated with 3-fold increase in HPV prevalence and current use of POCs is potentially linked to increased HPV incidence in short-term follow-up among pre- and

perimenopausal females over the age of 35 years. In this study, we sought to investigate the baseline cervical cytokine milieu of current versus non-current hormonal contraceptive users to see if the differences correlate with our epidemiologic observations in this population.

Materials and Methods

Study design

Baseline cervical secretion samples were taken from women enrolled in an observational prospective study assessing the natural history of HPV during the perimenopausal transition. Briefly, women aged 35 to 60 years were recruited from gynecological clinics associated with the Johns Hopkins Medical Institutions in Baltimore, MD. Exclusion criteria for the parent study included the following: (i) history of hysterectomy, organ transplant or HIV infection, (ii) currently pregnant, (iii) unable to provide informed consent, (iv) unwilling to provide contact information, (v) non-English speaking. The study protocols were reviewed and approved by the Institutional Review Board at Johns Hopkins Bloomberg School of Public Health, Baltimore, MD.

Standardized questionnaires were administered by trained interviewers at baseline and follow-up visits. Information was collected on (i) use of hormonal contraceptives including duration of use, time since last use, indications of use and brand names, (ii) demographic information, (iii) sexual behavioral characteristics, (iv) reproductive history, (v) smoking history, (vi) use of medications other than hormonal contraceptives, (vii) history of sexually transmitted infections.

HPV Detection

HPV DNA type-specific testing was done on cervical swab samples. DNA was extracted using the QIAamp DNA Blood Kit (Qiagen, Courtaboeuf, France) according to manufacturer's instructions with modification. After extraction, 8 µls of DNA (4% of total volume of extracted DNA) was amplified using the PGMY09/11 L1 consensus primer system, which amplified with high efficiency over 40 HPV genotypes known to infect the genital tract. Genotype discrimination was performed by hybridizing 40 µl of the PCR product to a membrane with immobilized probes targeting 37 HPV genotypes (HPV Linear Array Roche Molecular Systems, Roche Diagnostics, Indianapolis, IN).

Sample collection

Selection of samples for cytokine measurement

Samples from current hormonal contraceptive users were selected from subjects who (i) were HPV-negative at baseline, (ii) currently used hormonal contraceptives in the past 1 month, (iii) did not have abnormal Pap smears or genital infections in the past 6 months, (iv) did not currently use other exogenous sex hormones including hormone replacement therapy. Postmenopausal women were excluded from the study. Those who reported current hormonal contraceptive use but had last menstrual period more than 1 year ago were included in the selected samples (n=22) if (i) age <55 years, (ii) use of contraceptive (e.g. DMPA or Mirena) were related to cessation of periods, or (iii) use of HC was for "perimenopausal symptoms."

Samples from non-current hormonal contraceptive users were selected from those with non-current hormonal contraceptive use and those who were HPV-negative at baseline.

Non-current hormonal contraceptive users were excluded from selection if (i) status of last hormonal contraceptive use was <5 years ago or was missing, (ii) last menstrual period was >1 year ago, (iii) did not have abnormal Pap smears or genital infections in the past 6 months, (iv) did not currently use other exogenous sex hormones including hormone replacement therapy.

Current and non-current hormonal contraceptive users were randomly selected with 1:1 match on the following characteristics (i) age in 5-year categories, and (ii) last menstrual period in categories of days (1-14; 15-28, 28-40, 40-365). Last menstrual period was matched in categories among user pairs, except for 11 pairs for which no exact match were found and the closest category was used for matching instead.

Specimen processing and cytokine measurement

Protein for cytokine analysis was extracted from the ophthalmic sponges using previously described methods (29). Briefly, sponges were thawed at room temperature for 10 min and then weighed. Sponges were then inserted into a microcentrifuge tube containing a 0.2 lm filter (SpinX, Costar), equilibrated by adding 300 ul of extraction buffer (10 mg/ml aprotinin in phosphate buffered saline with 10% sodium azide) and incubated for 30 min at 4 ^{o}C, followed by centrifugation at 4 ^{o}C for 30 min at 14,000 rpm. After centrifugation, specimens were stored at 20 ^{o}C until the time of cytokine measurement. The following cytokines, chemokines, and growth factors were measured using a polystyrene non-magnetic bead-based multiplex assay according to the manufacturer's protocol (Bio-Rad, Hercules, CA): T_H1-related: IFN-γ, IL-2, IL-12, IL-15; T_H2-related: IL-4, IL-5, IL-9, IL-13; T_{reg}-related: IL-10, T_H17-related: IL-17, proinflammatory

cytokines and chemokines (eotaxin, IL-1β, IL-1rα, IL-6, IL-8, IP10, MCP1, MIP1α, MIP1β, RANTES, TNF-α), and growth factors (IL-7, G-CSF, GM-CSF, basic FGF, VEGF, PDGF- bb). Plates were read using a Luminex instrument (Bio-Rad, Hercules, CA). An eight point standard curve was created and fit using a five parameter logistic model. Specimens were tested across plates. Duplicates within plate and replicates across plates were tested to control for observed intra and inter-assay variability.

In order to control for inter-individual variability in immune marker measured due to differences in the amount of specimen collected, the total concentration of protein was measured in each specimen using a bicinchoninic acid (BCA) assay per manufacturer's protocol (Pierce, Rockford, IL). Specimens were diluted 1:10 and 1:100 in PBS and run in duplicate. Colorimetric detection of test specimens was normalized to background specimens that contain extraction buffer only. Total protein concentration was estimated using an eight point standard curve and is expressed as ug/ml.

Statistical analysis

Cytokine measurements

Cytokine concentrations that were outside detection range were managed as previously described (29). Specifically, those values that were below the limit of detection and completely outside the dynamic range and reported as undetectable by the Luminex instrument were assigned ½ the lowest measured value in the sample. Cytokine concentrations that were below the manufacturer's published limit of detection, but a concentration was estimated by the instrument from the standard curve, were assigned that imputed value. Cytokine concentrations that exceeded the upper detection limit of

detection and completely outside the dynamic range were coded as unobserved values. Cytokine that has more than 25% of values outside detection range were not included in analyses for comparison between exposure groups.

Comparison of cytokine measurements among hormonal contraceptive user groups

Protein-adjusted cytokine measurements were normalized by log transformation. Comparison of log-transformed levels of individual cytokines was made between matched pairs of current and non-current hormonal contraceptive users at baseline using paired t-test. Global comparison of cytokine measurements was made my assessing correlations between all pairwise combinations of cytokine measurements that were within detection limits. Spearman's rho, a non-parametric alternative to the correlation coefficient r, was used to calculate correlations. P values were adjusted by Sidak correction for multiple comparisons. Differences between correlation coefficients were tested by z-test of the Fisher's r to z-transformation of the Spearman rho correlation coefficient as previously published (30, 31).

Sensitivity analysis

Sensitivity analysis was performed with inclusion and exclusion of values that were either completely undetectable or outside the dynamic range of the assay. The observed pattern in correlation of immune markers by HPV status remained the same (results not shown).

Results

Population characteristics

A total of 105 pairs of samples from current and non-current hormonal contraceptive users matched in 5-year age groups were assessed. Mean ages of current and non-current hormonal contraceptive users were 42.4 (SD: 4.7 years) and 43.1 (SD: 4.7 years) years respectively. Most of the samples came from premenopausal women (75%). A majority of the tested population either never had abnormal Pap smear (58.6%) or had abnormal test result more than 1 year ago (39.5%). No significant difference was seen between current and non-current hormonal contraceptive users in terms of lifetime number of sexual partners, sexual activity in the past 6 months, history of colposcopy, marital status, parity or race (Table 5.1). Among current hormonal contraceptive users, 39% reported current use of combined oral contraceptives (COCs) and 47% had current use of progestin-only contraceptives (POCs) (Appendix. Appendix Table 5.2).

Cytokine measurements by hormonal contraceptive use

There was no difference between current and non-current hormonal contraceptive users in proportion of samples having values outside detection limits. Of the 27 cytokine markers measured, 4 had more than 25% of samples yielding values outside detection range, which included eotaxin, IL-8, IL-1ra, IL-5. Thus measurements of these 4 markers were not interpreted in comparison studies (Appendix. Appendix Table 5.1).

Levels of cytokines were not significantly different between current and non-current hormonal contraceptive users for the majority of markers assessed, except IP10 and IL1b for which marginal significant differences were noted . Current hormonal contraceptive

users had lower levels of IP10 (mean: 818.4 pg cytokine/mg protein; SD: 958.6 pg cytokine/mg protein) as compared to non-current users (mean: 1591.7 pg cytokine/mg protein; SD: 2302.2 pg cytokine/mg protein) (P=0.03). Slightly lower level of IL1b was observed among non-current users (mean: 81.9 pg cytokine/mg protein; SD: 111.8 pg cytokine/mg protein) than current users (mean: 95.8 pg cytokine/mg protein; SD: 158.4 pg cytokine/mg protein)(P=0.04) (Table 5.2).

Relative to non-current hormonal contraceptive matched subjects, current COC users had significantly lower levels of IL2 (P=0.02), IP10 (P=0.002) and MCP1 (P=0.01). In contrast, current POC users had higher level of IL1b (P=0.01), and lower levels of IL12 (P=0.03), IL13 (P=0.048), IL15 (P=0.048), as well as IP10 (P=0.02) than non-current hormonal contraceptive matched users (Table 5.3).

Correlations of cytokine measurements by hormonal contraceptive use

Significant positive correlations were observed among both current and non-current hormonal contraceptive users among immunoregulatory cytokines, including INF-γ, IL-2, IL-12, IL-4, IL-9, IL-10, IL-13, IL-15, IL-17, IL-7. However, among non-current users, significantly more proinflammatory cytokines were involved in positive correlations with immunoregulatory cytokines. IP10 was significantly more positively correlated with INF-γ, IL-12, IL-4, IL-9, IL-10, IL-13, IL-15, IL-17, in non-current users but not in current users. MCP1 was statistically significantly positively correlated with INF-γ, IL-2, IL-4, IL-15, IL-17, IL-7 in non-current users but such correlations were not observed in current users. Similarly, RANTES was statistically significantly correlated with INF-γ, IL-12, IL-4, IL-9, IL-10, IL-13, IL-15, IL-17, IL-7 in non-current users but not in current users

(Figure 5.1, Figure 5.2, Figure 5.3). The coefficients for the correlations noted above for IP10, MCP1 and RANTES were statistically significantly more negative (lower in magnitude) in current hormonal contraceptive users than non-current users (Figure 5.4). Correlations were also in general more negative (lower in magnitude) among other proinflammatory and immunoregulatory cytokines in current hormonal contraceptive users (Figure 5.4).

Correlations of cytokines among current COC and POC users involved mostly immunoregulatory cytokines. More significant correlations were observed among IL-15, IL-17, IL-9, IL-10, IL-12, and INF-γ in current COC users and current POC users. POC users appeared to show fewer significant correlations among immunoregulatory cytokines, as well as a suggestion of a more predominant Th2-skewed profile as evidenced by significant correlations between IL-4, IL-10, and IL-13 (Figure 5.5, Figure 5.6, Figure 5.7).

Compared to COC users, correlations were significantly weaker among immunoregulatory cytokines such as IL-9, IL-15 and IL-17, as well as among IL-5, IL-15, IL-17 in current POC users (Figure 5.8).

DISCUSSION

Cytokines are essential in mediating innate and cellular adaptive immune responses against viral infections. Data on correlates of mucosal immunity, particularly cytokines, in female human genital tract are limited in older women. In this study, we provided data on baseline cytokine milleu in women over the age of 35 years matched on age and

menstrual cycle, stratified on current use of hormonal contraceptives. Among healthy, HPV-negative women, while absolute levels of cytokines were not significantly associated with current use of any hormonal contraceptives, there was a significant lack of positive correlations among proinflammatory cytokines as well as between proinflammatory and immunoregulatory cytokines in current hormonal contraceptive users versus non-current users.

The lack of association between absolute cytokine levels and hormonal contraceptives could be explained in two ways. It is known that in asymptomatic women, even among those harboring HPV infection, there is in general a lack of measurable inflammatory response (18, 24, 25). Should hormonal contraceptive use have an effect on cervical levels of cytokines, the difference may be too small to detect in a small-scale study among healthy women with no observed genital inflammation. Furthermore cytokines are often produced by the same immune cell subsets or by cells that interact and regulate one another in a highly dynamic state, hence their biological levels and activities are context-specific (29, 32). Studies on individual levels of cytokines may therefore be subject to study variations in population characteristics, sampling techniques and analytic methodologies.

The need to study cytokines as an integrated network in addition to their individual levels becomes apparent when we consider the current body of work on cervical cytokines in women in relation to HPV status and hormonal contraceptive use. For instance, previous studies reported that IL-12 or IL12p70 levels were not associated with prevalent HPV

status (25, 26, 29), while two other studies reported higher levels of IL-12 in HPV-positive women relative to HPV-negative women (22, 25, 33). Lieberman et al suggested that IL-6 levels were reduced in women with incident HPV infections (32, reviewed in 25), while another study found that IL-6 levels tended to be elevated in HPV-positive women (33, reviewed in 25). Similar inconsistencies were reported in previous studies on cervical cytokines and hormonal contraceptive use. For example, IL-10 and IL-12 had been noted to be elevated in adolescent and young adult women on oral contraceptives (26, 27), but a recent study (22) did not replicate the findings. These inconsistencies suggest that comparing absolute levels of individual cytokines do not fully capture the interaction of cytokines with host and viral factors in the female genital tract.

Our study's findings were unique in highlighting the effect of current hormonal contraceptive use on the correlative patterns of cervical cytokines. Fluctuations of endogenous estradiol and progesterone have long been observed to influence mucosal immunity in the lower genital tract. Timing of menstrual cycle phase is associated with the concentrations of cyto-/chemokines in cervicovaginal secretions (36). Wira et al found that ectocervical and vaginal secretions exhibit a pattern of innate immune protection that is physiologically suppressed at mid-menstrual cycle (37, 38). Estradiol (E2) and progesterone influence the migration of immune cells, such as macrophages, dendritic cells (DC) as well as T and B cells, by affecting the expression of adhesion molecules and chemotactic factors (13, 37-38). These immune cells then signal the recruitment and activation of other cells of the innate and adaptive immune systems by the production of cytokines and chemokines. Exogenous sex hormones had also been demonstrated in previous studies to impair immune responses. Shust et al showed that the

ability of cervicovaginal lavage to inhibit HSV infection in vitro decreased significantly in oral contraceptive users compared with non-users (39, 40). Progesterone has been observed to suppress cell-mediated immune responses in vitro, including inhibiting cytotoxic T cell activity, reducing NK T cell function, and skewing cytokine production towards a general Th2 response (reviewed in 40). Mice treated with DMPA exhibited decreased levels of HSV-2 specific mucosal immune responses after intravaginal immunization with TK-HSV2 (40, 41). The effect of estrogen is more complicated, as the hormone can be either proinflammatory or anti-inflammatory depending on its concentrations (42). At high concentrations ($>10^{6}$M), estrogen was shown to inhibit cellular immunity by impairing cytotoxic T cell activity(43, 44) and decreasing migration of T cells and macrophages into the genital tract by downregulating expression of adhesion molecules including ICAM-1, VCAM-1 and E-selectins (28, 42, 44). At low concentrations ($<10^{-8}$M), estrogen induces TNF-α, IL-6 and IL-1β expression, inhibits Th2-type cytokines, and increases migration of leukocytes to the site of inflammation (28, 40, 42).

In our study, current hormonal contraceptive use was found to associate with significantly weaker positive correlations among proinflammatory cytokines, particularly IP10, MCP1, MIP1a and RANTES, as well as between proinflammatory and immunoregulatory cytokines that mediate Th1 and Th2 responses. In addition, overall cytokine correlations were more negative (lower in magnitude) among current hormonal contraceptive users than non-current users. The finding suggests that when active HPV infection is absent, exogenous sex hormones may dysregulate the baseline cervical cytokine links, resulting in less engagement of proinflammatory cytokines and

chemokines which are crucial in mediating innate and adaptive factors in mucosal immunity. It is known that the innate immune system, which serves as the first line of mucosal defense, is activated to create a pro-inflammatory microenvironment in the early stages of HPV infection (14). Immune cells are then recruited, initiating adaptive immune response. Clearance of HPV infection therefore relies on effective local Th1 cytokine expression (18, 21, 27, 43) and cellular immune response (44). Dysregulation of correlations among baseline proinflammatory and regulatory cytokines may likely hinder proper immune activation against active viral infection, leading to viral persistence. Alternatively, changes in baseline local immunity may potentially serve as a stimulus to reactivate viral infection which has stayed latent, resulting in an "incident" viral detection.

In this study, compared to COC users, correlations were significantly weaker among immunoregulatory cytokines such as IL-9, IL-15 and IL-17, as well as IL-5, IL-15, IL-17 in current POC users. The findings suggest differential impacts of estrogen versus progesterone on the cervical cytokine milieu. IL-5, IL-9, IL-13 areTh2-type are cytokines essential in mediating the pathogenesis of allergy and asthma (45-47), as well as in initiating immune responses against helminth parasites (48). These interleukins have been shown to associate with an increased HPV viral persistence (49) and high-risk HPV prevalence (7). IL-17, which is a pro-inflammatory cytokine, is also associated with allergic immune responses in the airway (50) as well as multiple autoimmune diseases (51). It was noted in a recent study by Marks et al that HPV infection in older women was associated with higher local concentrations of anti-inflammatory and allergy associated markers, with a shift in T-cell associated cytokine correlation from IL-2 to

eotaxin (29). Reduction in IL-2 and IL-12 levels were suggested in current COC and POC users, respectively, in our study. The significant decrease in correlations between proinflammatory and immunoregulatory cytokines in hormonal contraceptive users in our study further suggests that hormonal contraceptives may impair the basal mucosal immunoregulation against HPV by disrupting the overall cytokine balance in older women. The relative lack of correlations of allergy-related cytokines in current POC users versus COC users indicates possible difference in influences of estrogen and progesterone on local allergy-associated immune markers. It is unclear at this point how the effects observed may associate with duration of exogenous hormone exposure, and what different roles estrogen and progesterone may play in allergy-related immunity in the female genital tract. Further studies in those regards are warranted to better investigate the biologic roles of hormonal contraceptives in genital immunity in older women.

Our study was limited in power to assess the duration of contraceptive use on cervical immunity. It is known that only long-term, not short-term contraceptive use was associated with increased risk of HPV prevalence (7). Differences in cumulative or recent dosages of exogenous hormone intake may therefore have different impacts on cervical immunity. A few cytokines in our study population, including IL-1ra, IL-5, IL-8 and eotaxin, yielded >25% of values that were either undetectable or outside the dynamic range of the measurement assay and hence were excluded from final analysis and interpretation. While our study was short of informative data on those particular cytokines, our findings on the correlation patterns were robust with the rest of the markers other than a loss of statistical significance due to reduced sample sizes when

sensitivity analyses were done with inclusion and exclusion of the imputed cytokine values.

Our study has a number of strengths. Novel techniques were employed for adjusting sampling variability in this study. Total protein in cervical secretions was used to normalize measured levels of multiple cervical cytokines to produce accurate assessments across samples. Data and samples were obtained from a study that specifically examines HPV detection in a relatively focused age range across menopausal stages. With detailed information collected on demographic factors and the recency of hormonal contraceptive use, we were able to assess cervical cytokine profile among women matched on age and days of menstrual cycle, accounting for potential confounding due to endogenous hormonal fluctuations.

In summary, our findings suggested that current hormonal contraceptive use diminishes the positive correlations among proinflammatory and immunoregulatory cytokines in healthy women over the age of 35 years. This effect on baseline cytokine milieu hints at potential impairment in eliciting proper cytokine release and immune activation in the face of active viral infection.

Our study was a cross-sectional analysis of cervical cytokine profile. Other factors that are subject to hormonal influence, such as immune cells and vaginal flora, may contribute to changes of mucosal defense, for which more studies are warranted for better understanding of exogenous hormonal effect on local immunity in the female genital tract.

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TABLES AND FIGURES

Table 5.1. Baseline characteristics of current and non-current hormonal contraceptive users with measurement of cervical cytokine levels

Characteristics at baseline	Total N=210 (col %)	Samples from current* hormonal contraceptive users n= 105 (col %)	Samples from non-current hormonal contraceptive users n=105 (col%)	P
Demographic characteristics				
Age	Mean: 42.8. SD: 4.7 Median: 43. IQR: 7	Mean: 42.4. SD: 4.7 Median: 42. IQR: 7	Mean: 43.1. SD: 4.7 Median: 43. IQR: 9	0.31
Race				
Caucasian	139 (66.2)	67 (63.8)	72 (68.6)	0.30
African American	58 (27.6)	33 (31.4)	25 (23.8)	
Others	13 (6.2)	5 (4.8)	8 (7.6)	
Marital status				
Married	119 (56.7)	52 (49.5)	67 (63.8)	0.91
Single	57 (27.1)	28 (35.2)	20 (19.0)	
Divorced/separated/widowed	34 (16.2)	16 (15.2)	18 (17.1)	
Parity				0.75
0 or 1	64 (30.5)	31 (29.5)	33 (31.4)	
2	63 (30.0)	39 (37.1)	44 (22.9)	
>2	83 (39.5)	35 (33.3)	48 (45.7)	
Sexual behavioral characteristics				
Lifetime number of sexual partners				0.63
1	21 (10.0)	9 (8.6)	12 (11.4)	
2-4	43 (20.5)	20 (19.0)	23 (21.9)	
5-6	38 (18.1)	58 (55.2)	40 (38.1)	
>10	48 (22.9)	18 (17.1)	30 (28.6)	

Sexual activity in last 6 months				
No sex	30 (14.3)	19 (18.1)	11 (10.5)	0.19
Have sex but no new sexual partner	155 (73.8)	84 (80.0)	81 (77.1)	
Have sex with new sexual partner	25 (11.9)	12 (11.4)	13 (12.4)	
Ever undergone colposcopy				
No	171 (81.4)	87 (82.9)	84 (80.0)	0.86
Yes	39 (18.6)	18 (17.1)	21 (20.0)	
Time since last abnormal pap smear				
Never had abnormal pap smear	112 (53.3)	48 (45.7)	64 (61.0)	0.54
<1 year ago	1 (0.5)	0 (0)	0 (0)	
2-5 years ago	45 (21.4)	30 (28.6)	15 (14.3)	
≥6 years ago	52 (24.8)	27 (25.7)	27 (25.7)	

*Current users included those with last use ≤ 1 month ago. Non-current controls were selected from those with no use or last use of hormonal contraceptives >5 years ago.

Table 5.2: Distribution of mean cytokine measurements by hormonal contraceptive use

	Total samples (N=210)		Protein-adjusted cytokine levels of matched pairs* (pg cytokine/mg protein)				
			Current HC users (n=105)		Non-current HC controls (n=105)		
Cytokine	**Mean**	**sd**	**Mean**	**sd**	**Mean**	**sd**	**P****
IL1b	88.9	137.2	95.8	158.4	81.9	111.8	0.04
IL2	4.8	12.1	3.0	4.8	6.7	16.3	0.05
IL4	0.4	0.2	0.3	0.2	0.4	0.3	0.89
IL6	59.6	82.2	60.2	75.2	59.1	89.0	0.17
IL7	4.3	4.7	3.4	3.1	5.2	5.8	0.09
IL9	6.8	5.8	6.6	5.5	7.0	6.1	0.88
IL10	8.3	7.7	8.1	7.2	8.6	8.2	0.96
IL12	36.5	30.8	34.9	27.7	38.0	33.7	0.86
IL13	5.3	4.7	5.0	3.9	5.6	5.3	0.90
IL15	3.3	3.9	2.9	3.2	3.6	4.4	0.66
IL17	9.2	9.1	8.7	6.4	9.8	11.2	0.66
GCSF	590.2	846.1	538.3	532.8	638.1	1057.4	0.50
GMCSF	214.9	214.2	212.7	207.8	217.2	221.3	0.71
IFN-γ	30.7	22.2	29.6	18.9	31.9	25.1	0.93
IP10	1205.1	1801.2	818.4	958.6	1591.7	2302.2	0.03
MCP1	80.6	127.2	66.6	113.8	94.1	138.1	0.43
MIP1a	4.0	5.6	4.0	5.2	4.0	6.0	0.45
MIP1b	68.2	96.2	69.8	75.8	66.6	113.4	0.07
RANTES	17.8	37.8	18.4	26.5	17.2	46.9	0.06
TNF-α	6.9	5.2	6.6	4.2	7.2	6.1	0.83
VEGF	450.0	361.8	448.0	320.6	452.0	401.4	0.30
FGF	40.9	54.1	38.3	53.1	43.6	55.3	0.60
PDGF	62.5	56.5	62.3	60.1	62.7	52.9	0.71

* Samples from current hormonal contraceptive (HC) users and non -current contraceptive users were matched by 5-year age categories and closest categories of days of last menstrual period.
**Paired t-test of log-transformed cytokine measurements

Table 5.3: Distribution of mean cytokine measurements by combined oral contraceptive use and progestin-only contraceptive use

	Total samples (N=210)		Protein-adjusted cytokine levels of matched pairs* (pg cytokine/mg protein)									
			Current combined oral contraceptive users in matched pairs (n=41)		Non-current contraceptive users in matched pairs (n=41)			Current progestin-only contraceptive users (n=44)		Non-current contraceptive users in matched pairs (n=44)		
Cytokine	**Mean**	**sd**	**Mean**	**sd**	**Mean**	**sd**	**P*****	**Mean**	**sd**	**Mean**	**sd**	**P****
IL1b	88.9	137.2	86.4	159.1	92.4	124.1	0.86	101.9	174.2	65.3	98.5	0.01
IL2	4.8	12.1	2.9	3.1	7.4	14.1	0.02	3.2	6.6	6.1	18.7	0.34
IL4	0.4	0.2	0.3	0.2	0.4	0.4	0.42	0.3	0.2	0.3	0.2	0.20
IL6	59.6	82.2	57.5	73.1	45.2	64.1	0.46	58.9	66.9	65.2	111.4	0.81
IL7	4.3	4.7	3.6	2.7	5.9	6.4	0.05	3.0	3.4	4.5	4.2	0.09
IL9	6.8	5.8	6.5	3.3	7.6	7.4	0.39	5.8	6.0	6.5	4.2	0.50
IL10	8.3	7.7	9.1	8.8	8.9	10.7	0.91	6.2	4.4	8.4	6.4	0.08
IL12	36.5	30.8	39.6	31.2	38.7	42.4	0.92	27.1	18.0	36.6	24.9	0.03
IL13	5.3	4.7	5.6	3.4	5.9	7.0	0.81	3.9	2.8	5.3	3.6	0.048
IL15	3.3	3.9	3.2	2.6	3.9	5.3	0.50	2.1	2.3	3.6	4.0	0.048
IL17	9.2	9.1	8.6	4.4	11.8	16.2	0.23	7.3	6.6	8.5	5.6	0.43
GCSF	590.2	846.1	578.4	483.8	563.3	638.4	0.43	495.2	568.7	679.8	1390.3	0.55
GMCSF	214.9	214.2	208.8	119.9	233.9	308.2	0.62	169.8	188.9	202.8	124.1	0.35
IFN-γ	30.7	22.2	31.7	15.5	33.5	30.1	0.74	24.2	14.3	29.8	18.7	0.12
IP10	1205.1	1801.2	701.5	874.1	1659.7	1832.1	0.002	799.2	809.6	1733.0	2688.4	0.02
MCP1	80.6	127.2	45.8	63.0	119.5	163.4	0.01	88.4	159.4	85.9	119.3	0.95
MIP1a	4.0	5.6	3.4	4.2	3.2	5.0	0.87	3.4	3.9	5.1	7.6	0.20
MIP1b	68.2	96.2	78.3	85.4	43.2	47.3	0.05	53.0	61.5	92.1	159.0	0.24
RANTES	17.8	37.8	16.8	24.6	10.2	13.4	0.22	19.4	30.9	23.4	68.8	0.66
TNF-α	6.9	5.2	6.7	4.0	7.9	7.6	0.41	5.7	3.5	6.8	4.8	0.28

VEGF	450.0	361.8	498.7	351.4	459.4	475.3	0.79	362.6	254.7	433.5	324.3	0.22
FGF	40.9	54.1	38.6	53.0	45.3	64.6	0.61	30.5	39.7	41.6	45.0	0.22
PDGF	62.5	56.5	68.6	80.5	63.2	51.0	0.72	51.6	40.3	65.5	47.6	0.14

* Samples from current hormonal contraceptive (HC) users and non-current contraceptive users were matched in 5-year age categories and closest categories of days of last menstrual period.
**Paired t-test of log-transformed cytokine measurements.

Legend	
	Correlation coefficient was statistically significant (p value ≤0.01) after adjusting for multiple comparisons.
	Correlation coefficient was statistically significant (p value ≤0.05) after adjusting for multiple comparisons.
	Correlation coefficient was not statistically significant (p value >0.05) after adjusting for multiple comparisons.

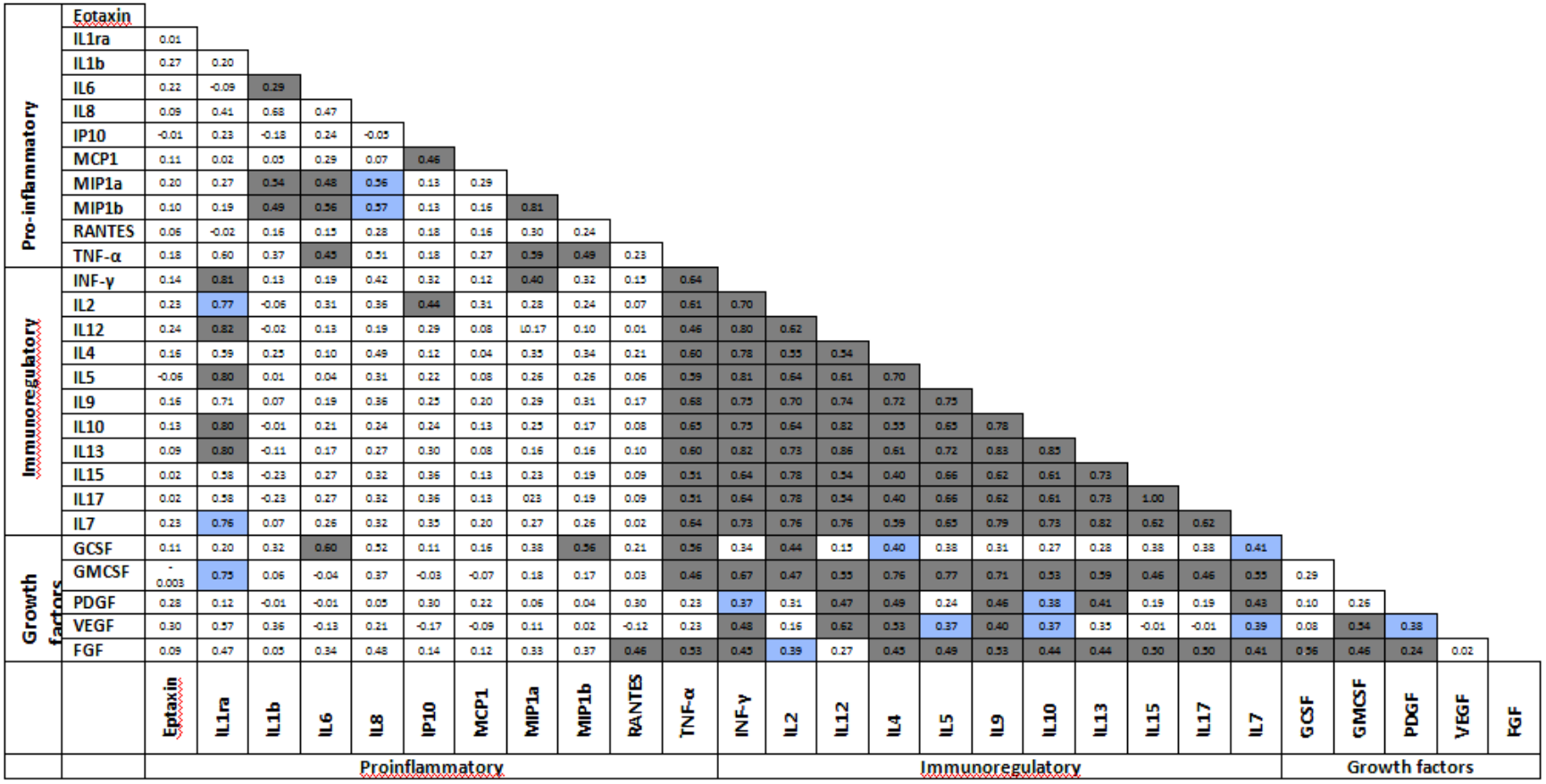

Figure 5.1. Pairwise Spearman's rank correlations of cytokine measurements in current hormonal contraceptive users at baseline

Legend	
	Correlation coefficient was statistically significant (p value ≤0.01) after adjusting for multiple comparisons.
	Correlation coefficient was statistically significant (p value ≤0.05) after adjusting for multiple comparisons.
	Correlation coefficient was not statistically significant (p value >0.05) after adjusting for multiple comparisons.

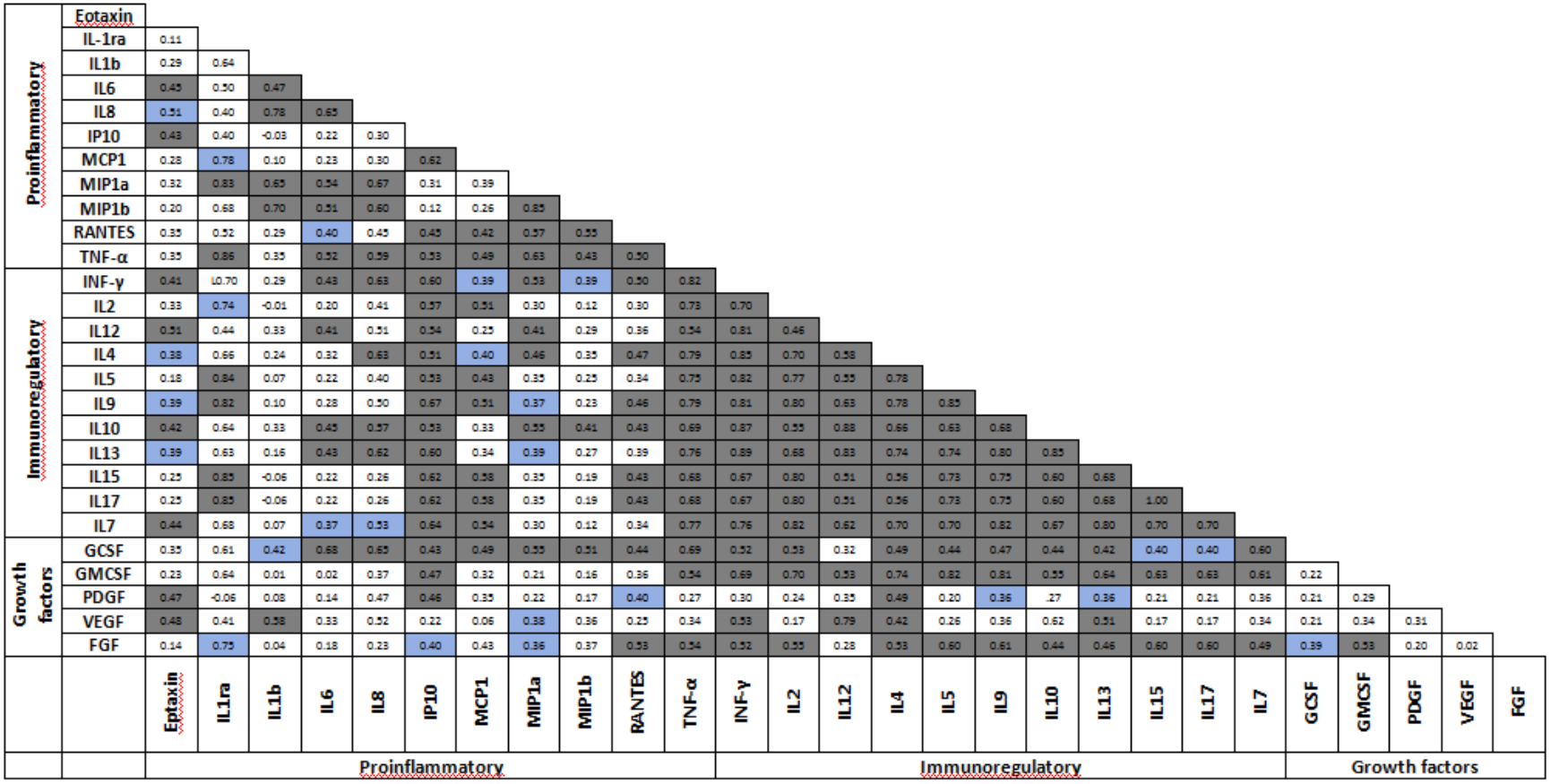

Figure 5.2. Baseline pairwise Spearman's rank correlations of cytokine measurements in non-current hormonal contraceptive users

Legend	
	Correlation coefficient was statistically significant in BOTH current and non-current hormonal contraceptive users.
	Correlation coefficient was statistically significant ONLY IN CURRENT hormonal contraceptive users.
	Correlation coefficient was statistically significant ONLY IN NON-CURRENT hormonal contraceptive users.
	Correlation coefficient was NOT statistically significant (p value >0.05) in either current or non-current hormonal contraceptive users.

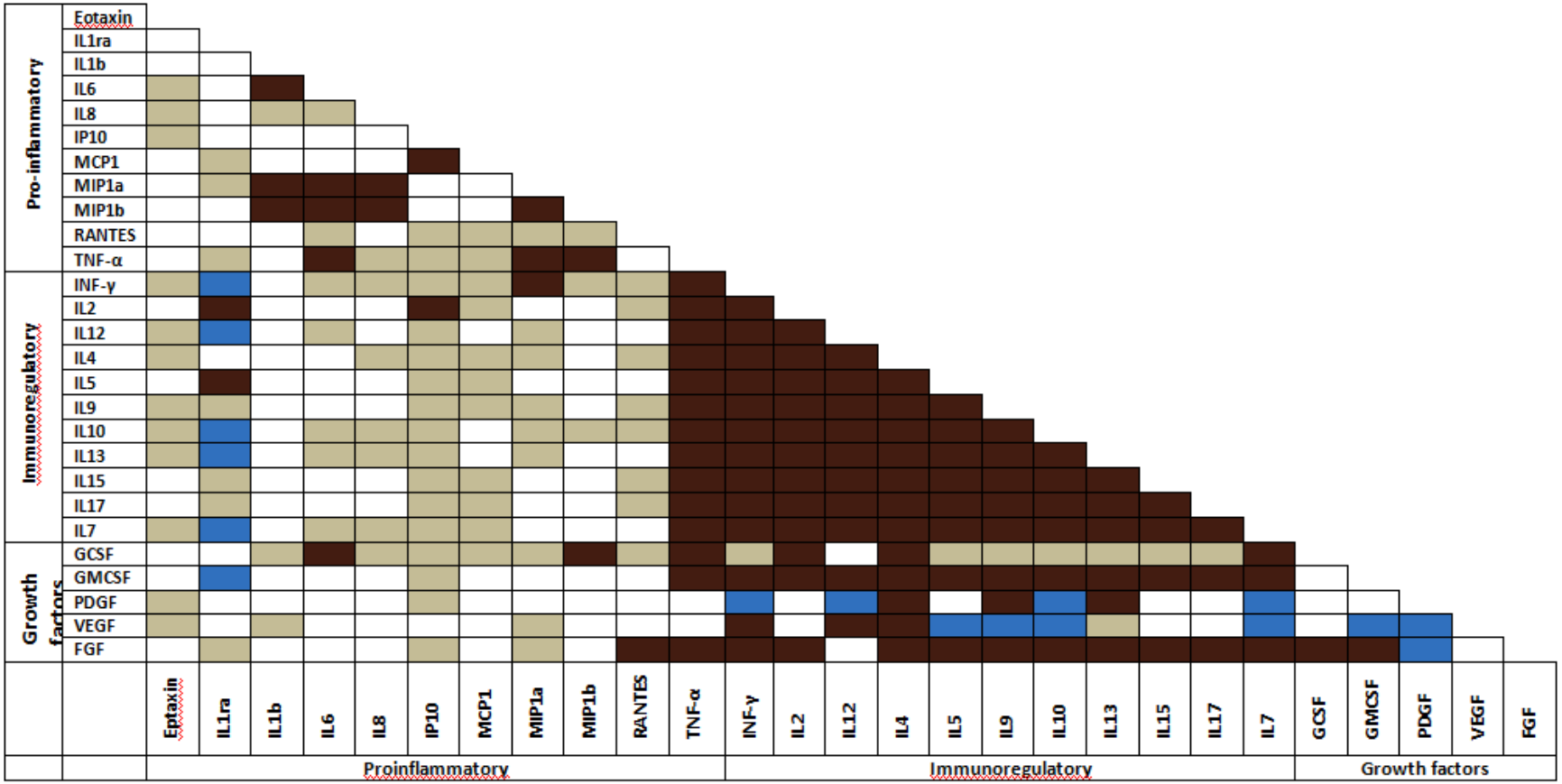

Figure 5.3. Baseline pairwise Spearman's rank correlations of cytokine measurements in current and non-current hormonal contraceptive users

p value noted in cell: Difference of correlation coefficients between current and non-current hormonal contraceptive users were statistically significant (p<0.05)
Correlation coefficient was more negative (lower in magnitude) among current hormonal contraceptive users
Correlation coefficient was more positive among current hormonal contraceptive users

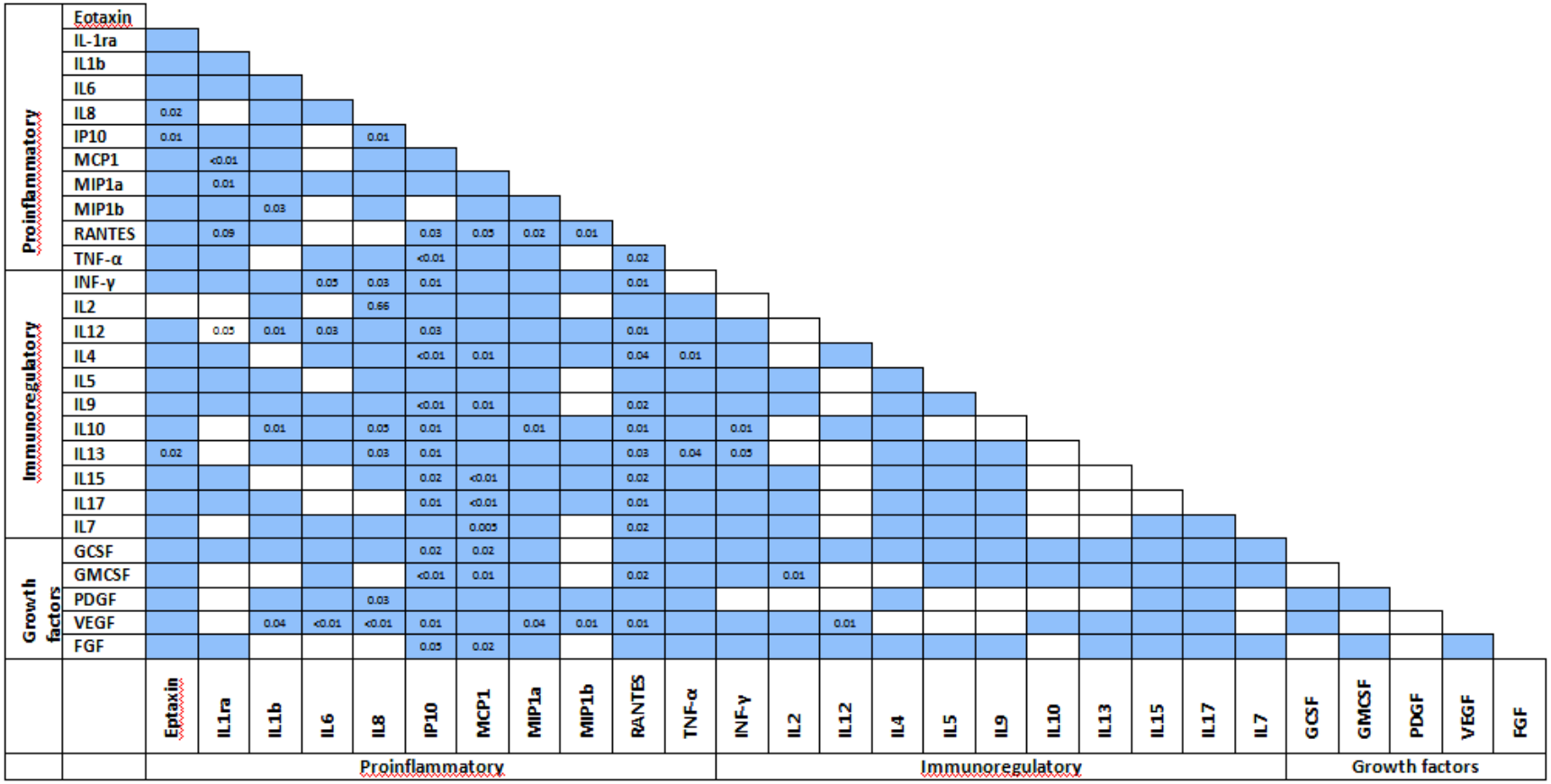

Figure 5.4. Heterogeneity of correlation coefficients by different cytokines: current hormonal contraceptive users versus non-current users

Legend	
	Correlation coefficient was statistically significant (p value ≤0.01) after adjusting for multiple comparisons.
	Correlation coefficient was statistically significant (p value ≤0.05) after adjusting for multiple comparisons.
	Correlation coefficient was not statistically significant (p value >0.05) after adjusting for multiple comparisons.

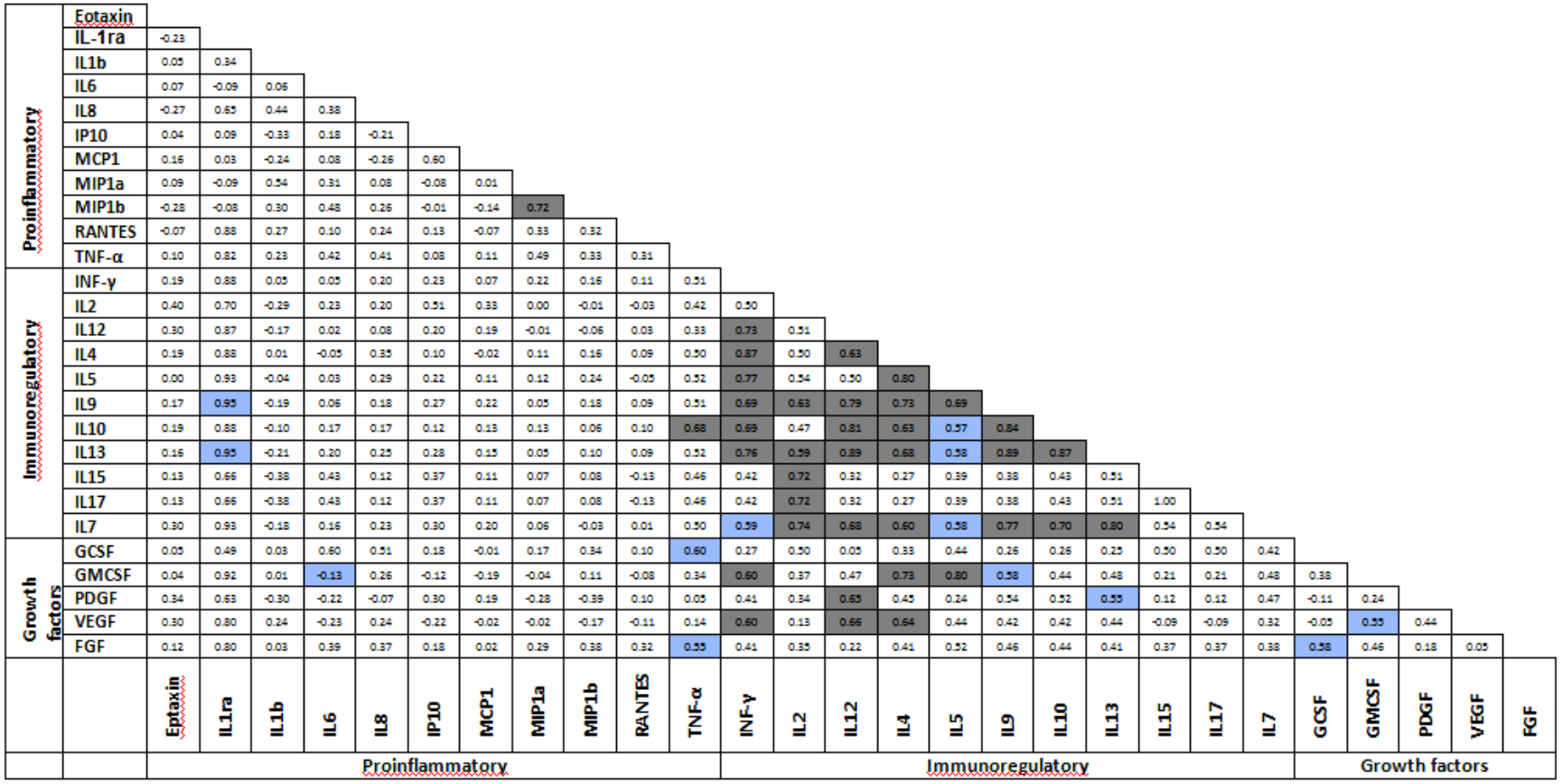

Figure 5.5. Baseline pairwise Spearman's rank correlations of cytokine measurements in current progestin-only contraceptive users

Legend	
	Correlation coefficient was statistically significant (p value ≤0.01) after adjusting for multiple comparisons.
	Correlation coefficient was statistically significant (p value ≤0.05) after adjusting for multiple comparisons.
	Correlation coefficient was not statistically significant (p value >0.05) after adjusting for multiple comparisons.

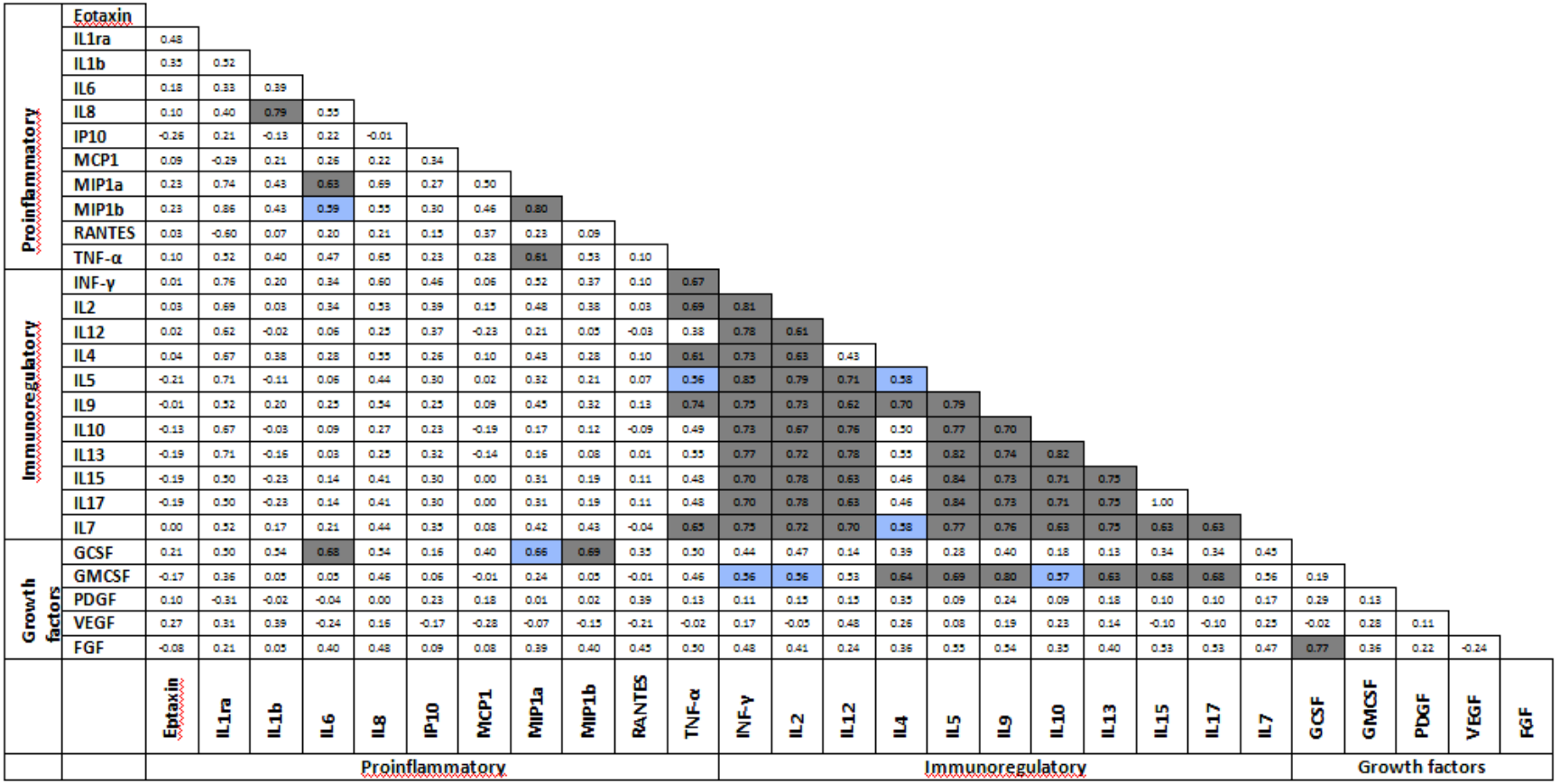

Figure 5.6. Baseline pairwise Spearman's rank correlations of cytokine measurements in current combined oral contraceptive users

Legend (COC: combined oral contraceptive; POC: progestin-only contraceptive; HC: hormonal contraceptive)	
	Correlation coefficient was statistically significant in BOTH comparisons: current COC vs. matched non-current HC users and current POC vs. matched non-current HC users.
	Correlation coefficient was statistically significant ONLY in comparison between current COC users vs. non-current HC users.
	Correlation coefficient was statistically significant ONLY in comparison between current POC users vs. non-current HC users.
	Correlation coefficient was not statistically significant in either comparison.

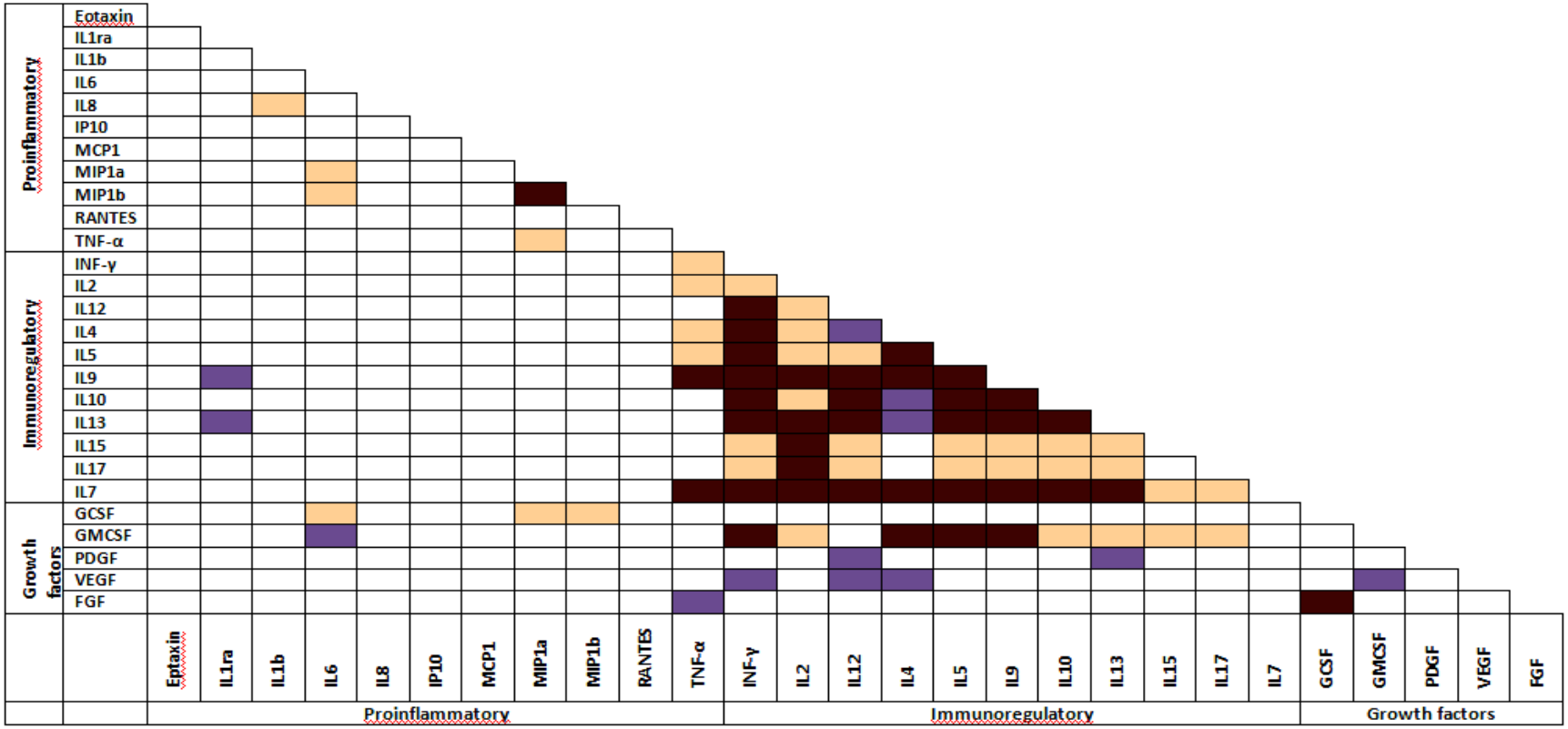

Figure 5.7. Baseline pairwise spearman rank correlations of cytokine measurements in current combined oral contraceptive users and progestin-only contraceptive users

P value noted in cell: Difference of correlation coefficients between current combined oral contraceptive users and progestin-only contraceptive users were statistically significant (p<0.05)	
	Correlation coefficient was more negative (lower in magnitude) among current combined oral contraceptive users
	Correlation coefficient was more positive among current combined oral contraceptive users

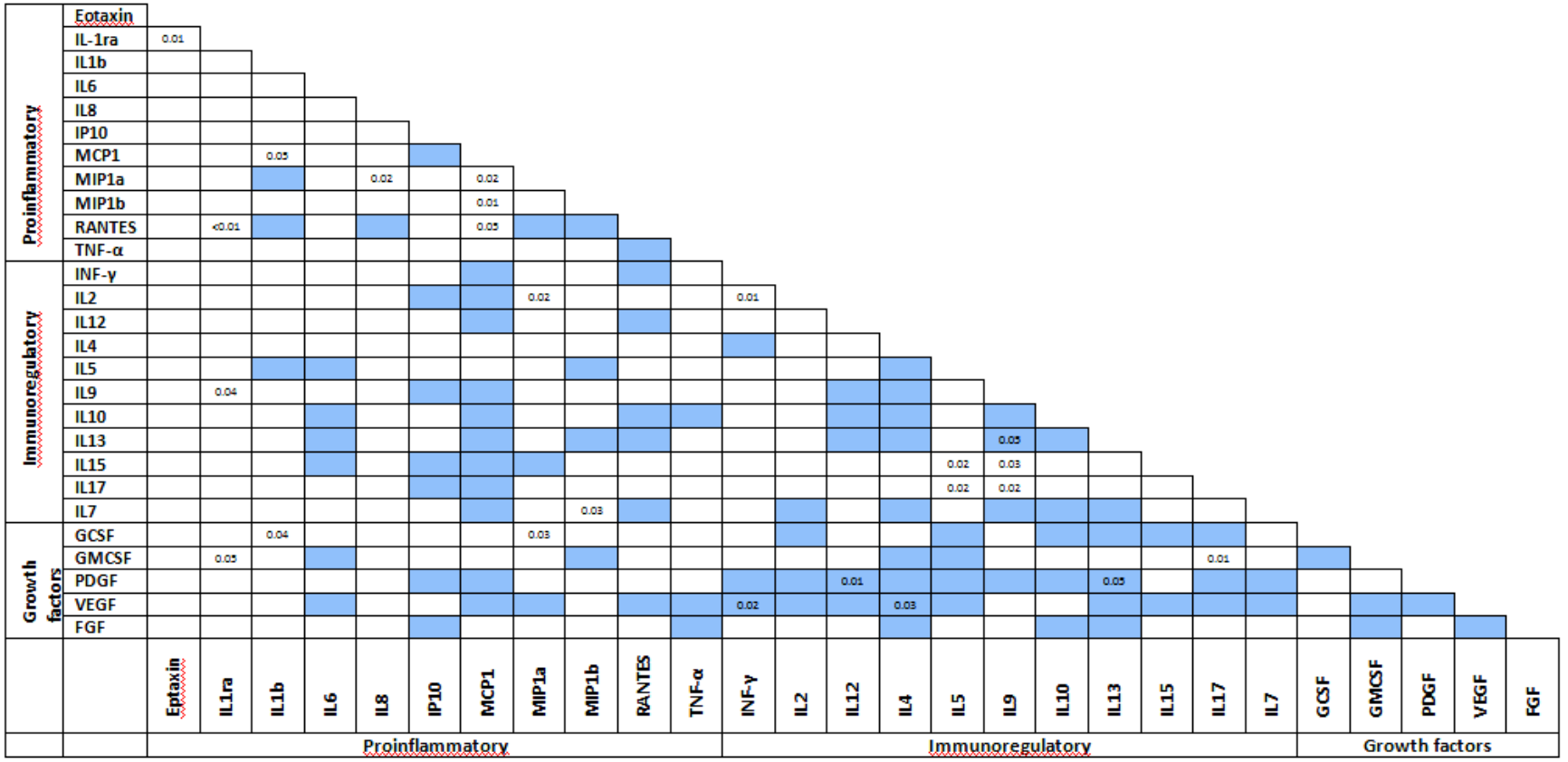

Figure 5.8. Heterogeneity of correlation coefficients by different cytokines: current combined oral contraceptive users versus progestin-only contraceptive users

CHAPTER 6:

Discussion and Conclusions

by

Thing Rinda Soong

SYNOPSIS OF DISSERTATION RESEARCH

The overall objective of this thesis research was to evaluate the association of hormonal contraceptive use with human papillomavirus (HPV) detection among pre- and perimenopausal women in the U.S. This objective was addressed by the following 3 study aims. A conceptual model summarizing the hypotheses supported by our study findings is shown in figure 6.1 (Figure 6.1).

<u>Aim 1:</u> To determine the association of hormonal contraceptive use with the prevalence of HPV DNA detection in pre- and perimenopausal women (N=530)

Women aged from 35-54 years (mean age was 44.2 years (SD: 5.4 years) were included in analyses. Relative to never progestin-only contraceptive (POC) users, more than 5 years' use of POCs was associated with increased prevalence of any HPV [adjusted prevalence ratio (aPR): 3.16 (95% CI: 1.82-5.48)] and HR-HPV [aPR: 4.26 (95% CI: 1.60-11.30)]. Current POC use was positively associated with HR-HPV (aPR: 2.44 (0.99-5.99) and any HPV (aPR: 1.58 (0.94-2.65) with the estimates bordering on statistical significance. No significant association was seen between prevalent HPV infection and the recency or duration of oral contraceptive (OC) use in this study cohort.

Our findings are coherent with those of pooled analyses to date, which revealed neither a strong positive or negative association between OC use and HPV prevalent infection (1, 2). Studies that reported positive associations between HPV infection and OCs or combined oral contraceptives (COCs) were mostly conducted in younger women below the age of 40 years (3-10).

Prevalence of HPV infection is driven by incidence and duration. The difference in associations observed in our cohort of older women compared to previous studies conducted in younger women may potentially be due to differences in sexual behavioral profiles (11), and/or differential effects by types of exogenous female hormones on cervical immunity, resulting in differences in proportions of incident vs. transient vs. persistent detection at baseline. POC use may have impact on the natural history of HPV infection in older women after controlling for sexual behavioral risk factors. A longitudinal study was performed (Aim 2) to better define this association and its clinical significance.

<u>Aim 2:</u> To estimate the association between hormonal contraceptive use and incident HPV DNA detection in pre- and perimenopausal women

Median follow-up time was 19.1 months with an interquartile range of 7.3 months. Relative to never users of POCs, current use of POCs was associated with new HR-HPV detection [adjusted odds ratio (aOR): 3.24 (95% CI: 1.37-7.65). No statistically significant association was detected between incident HPV detection and current use of OCs, nor was there significant association observed between duration of use of overall hormonal contraceptives and OCs.

COC was proposed as a risk factor of HPV prevalence in younger female populations (3-10) with the association proposed to be driven by COC's effect on HPV persistence and not on acquisition (12). The majority of OCs belongs to the category of COCs (13, 14). Our analyses on short-term prospective data revealed that current POC use was potentially linked to increased incident HPV detection. Secondary analysis showed that

no significant difference in proportions of persistent prevalent/incident HPV detection at 6 months was seen in association with any hormonal contraceptive use.

<u>Aim 3:</u> To describe and compare differences in the host cervical cytokine profiles among contraceptive users and non-users in pre- and perimenopausal women

We observed differences in cytokine correlation patterns between current and non-current hormonal contraceptive users, as well as between current POC and COC users. In non-current hormonal contraceptive users, there were significantly more positive correlations detected among proinflammatory cytokines, as well as between proinflammatory and immunoregulatory cytokines. Compared to COC users, POC users appeared to show fewer significant correlations among immunoregulatory cytokines, as well as a suggestion of a more predominant Th2-skewed profile as evidenced by significant correlations between IL-4, IL-10, and IL-13. The significant decrease in correlations between proinflammatory and immunoregulatory cytokines in hormonal contraceptive users in our study suggests that hormonal contraceptives may impair the basal mucosal immunoregulation against HPV by disrupting the overall cytokine balance in older women.

STRENGTHS

Our epidemiologic studies have a number of strengths. Analyses were based on a unique study that collected extensive data on the status, types, recency and duration of hormonal contraceptive use, allowing more specific characterization of exogenous sex hormone

exposure and its association with HPV infection. In addition, information was collected on days of menstrual cycle which reflected endogenous female sex hormonal levels for assessment. Furthermore, detailed information was also obtained on sexual behavior and other risk factors to evaluate correlates that may confound the associations between HPV prevalence and hormonal contraceptive use in older women.

Our study population also had a baseline racial demographic, and sexual behavioral characteristics that was generally representative of the U.S. population in this age range (11, 15, 16), making our findings relevant to the general population. Participants in the study were recruited from women presenting to gynecological clinics who are routinely screened for HPV. One of the implications of identifying determinants of HPV detection other than sexual behavioral factors, such as hormonal contraceptive use, is to improve testing-associated counseling of women who are being screened for cervical cancer. Given that, our study results are applicable to our target population.

LIMITATIONS

Our study has its limitations. Causal inference may be restricted in cross-sectional analyses performed for Aims 1 and 3. HPV DNA detection and cytokine levels were assessed at only one single time-point at baseline, making inferences challenging. Longitudinal association between hormonal contraceptive use and incident HPV detection was further assessed in Aim 2 using short-term prospective data. Analyses are

being planned and performed with 2 years of follow-up data to evaluate the impact of hormonal contraceptive use on HPV persistence and clearance.

Similar to other studies on HPV natural history, this thesis research may suffer from left truncation bias due to unobserved HPV DNA status prior to study entry, as well as interval sampling bias due to unobserved HPV DNA status between study visits (17), resulting in cases misclassified as "new" infections, as shown and explained in figure 6.2 (Figure 6.2). Studies have noted that point prevalence estimates can generally underestimate cumulative prevalence by about 20% (18-24). If exogenous hormonal exposure, e.g. POCs, causes increase in HPV detection by contributing more to transient DNA detection rather than persistent HPV infection, then our study estimates of associations are likely to be underestimated due to more "missed" detection related to interval censoring among the contraceptive users than in the non-users. On the other hand, if exogenous hormonal exposure causes increase in HPV detection by raising risk of persistent infection, then the bias impact on the association will likely be less in magnitude.

The measurement of exposure to hormonal contraceptives was based on self-report. There is a possibility of under-reporting of use as well as differential recall of use related to underlying conditions. These would introduce misclassification of exposure which would bias the estimates of associations. Previous studies comparing self-reported contraceptive use to clinical records in women aged 20 to 42 years have shown relatively high agreement, especially with regard to the duration and hormonal composition of

current and recent use (25-26). The amount of misclassification introduced by discrepancies in self-report of recent use of hormonal contraceptives is thus relatively small, which will likely not obscure the directionality of associations obtained from this study.

PUBLIC HEALTH IMPLICATIONS

Persistent HPV infection of high-risk HPV (HR-HPV) subtypes is a necessary cause of cervical precancer and cancer development. Although prevalence of HPV and HR-HPV is observed to be lower in older age groups in the U.S. (27, 28), the rate of cervical cancer increases steadily by age after correcting for hysterectomy. While the uncorrected cervical cancer rates plateaued at ages 40-44 years, the incidence rate after correcting for hysterectomy was shown to increase till the age of 60-69 years (28). Proper screening for cervical cancer and accurate interpretation of screening results is a clinically significant issue in women over the age of 35 years.

Women aged 30 to 65 years are currently recommended to be screened with either (i) (preferred) cytology and HPV testing every 5 years, or (ii) (acceptable) cytology alone every 3 years. (29, 30). The addition of HPV testing to cytology has been shown in studies to result in increased detection of CIN3 as well as cervical adenocarcinoma with concomitant decrease of precancerous and cancer lesions detected in subsequent screening (31-35). Recently, interim guidelines for a primary HPV screening strategy

have been proposed after the U.S. Food and Drug Administration approved the Roche Cobas HPV test for primary screening (36).

Application of HPV testing to women older than 30 years but not below also results in higher test specificity as HPV prevalence among women 30 years and older is lower than among younger women (31). However, interpretation of HPV test results can be problematic in women aged >35 years as there is a loss of correlation observed between HR-HPV test results and cytologic abnormalities as age increases, likely due to increased proportion of ASCUS and reduced prevalence of HR-HPV detected in older women (37).

An optimal screening strategy should be informative on the nature of a positive screening result, i.e. whether the result represents a transient HPV detection or an indicator with clinical potential to progress to cancerous lesions, so as to avoid unnecessary treatment and anxiety. Current knowledge is limited among older women aged 35 or above in (i) determinants of persistent high-risk HPV infection, (ii) biologic roles that risk factors of HPV infection may play in the host; (iii) and whether these HPV detections have clinical relevance to developing precancerous or cancerous lesions.

Our study findings addressed certain aspects of these knowledge gaps by improving current understanding between hormonal contraceptive use and HPV detection among older women aged 35-54 years independent of sexual behavior. Older women who are on long-term and current use of POCs may carry an increased risk of prevalent HR-HPV and any HPV detection, as well as incident HPV detection. These findings may have been

related to difference in cervical cytokine milieu observed in current and non-current hormonal contraceptive users, as well as between current POC users and current COC users. In contrast to previously published findings which focused mostly in younger women, no statistically significant associations were detected in our cohort between HPV detection and use of overall hormonal contraceptives and combined oral contraceptives. The observed differences hinted at potential differential biologic effects of exogenous hormones on local immunity and/or in different age groups that would warrant follow-up studies.

Findings obtained from this research will help us to generate hypotheses and future studies to further define natural history of HPV infection in older females. Admittedly, further studies of a larger scale are needed to address the knowledge gaps in HPV natural history to facilitate establishment of an optimal screening strategy in this older age group.

FUTURE DIRECTIONS AND CONCLUSIONS

Future follow-up studies are needed to address the following questions:

Our epidemiologic study findings suggested that long-term and current use of POC's was associated with increased HPV prevalence and incident HPV detection.

(i) Does incident HPV detection in this cohort represent newly acquired infection, recurrent infection of a previously cleared HPV subtype, or reactivation of a latent infection?

Shorter interval follow-up would help to differentiate types of incident HPV detection with regard to acquisition or viral reactivation/fluctuation of viral load. HPV genome full sequencing can also be incorporated to assess whether the detection reflects a newly acquired HPV subtype or a re-emerging detection of a persistent or cleared infection.

(ii) How would HPV type-specific serostatus of vaccinated and unvaccinated older women impact on HPV detection with respect to hormonal contraceptive use?

Studies need to be done with data collected on HPV antibody profile of women in different age strata and vaccine status in conjunction with other correlates of HPV infection including sexual behavioral risk factors, reproductive characteristics, exogenous and endogenous female sex profiles.

(iii) Does increased HPV prevalence detected in this cohort translate to higher risk of developing precancerous and cancerous lesions?

Prospective studies are needed with long-term follow-up to assess the rates of persistence and clearance, as well as incident abnormal cytologies and incident invasive cancer. These long-term outcomes would need to be interpreted with regard to types, recency and duration of hormonal contraceptive use, as well as the types of HPV detection, i.e. whether the HPV detection is newly acquired, or is a re-emerged detection of a persistent infection or a previously-cleared infection.

Studies of larger sample size would also be needed to assess long-term outcome with respect to different strata of hormonal contraceptive use in terms of type, duration and recency.

Our laboratory study showed that current hormonal contraceptive use at baseline was associated with weaker correlations among proinflammatory and immunoregulatory cytokines.

(iv) How wound the difference be interpreted in the context of immune cells and other clinical characteristics, and how would that tie to change of HPV status over time?

Specific cytokine-secreting subpopulations in correlation with cytokine profiles would need to be identified, e.g. via multiparameter flow cytometric assays and analyzed with multivariate techniques. A longitudinal study would be helpful to study trajectories of immune profile over time with change of HPV status among older women.

CONCLUSIONS

This dissertation showed that long-term and current use of POCs but not OCs may be associated with increased risk of prevalent and incident HPV infection independent of sexual behavior among pre- and perimenopausal women aged over 35 years with normal cervical cytology. Our laboratory study showed differences in cytokine profiles between current and non-current hormonal contraceptive users as well as between current POC and COC users in HPV-negative women. The study contributes to better understanding of the effect of hormonal contraceptive use on HPV detection in women in this age group. Further studies are warranted to better define the biologic role(s) of exogenous hormones in the natural history of HPV infection among older women.

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FIGURES

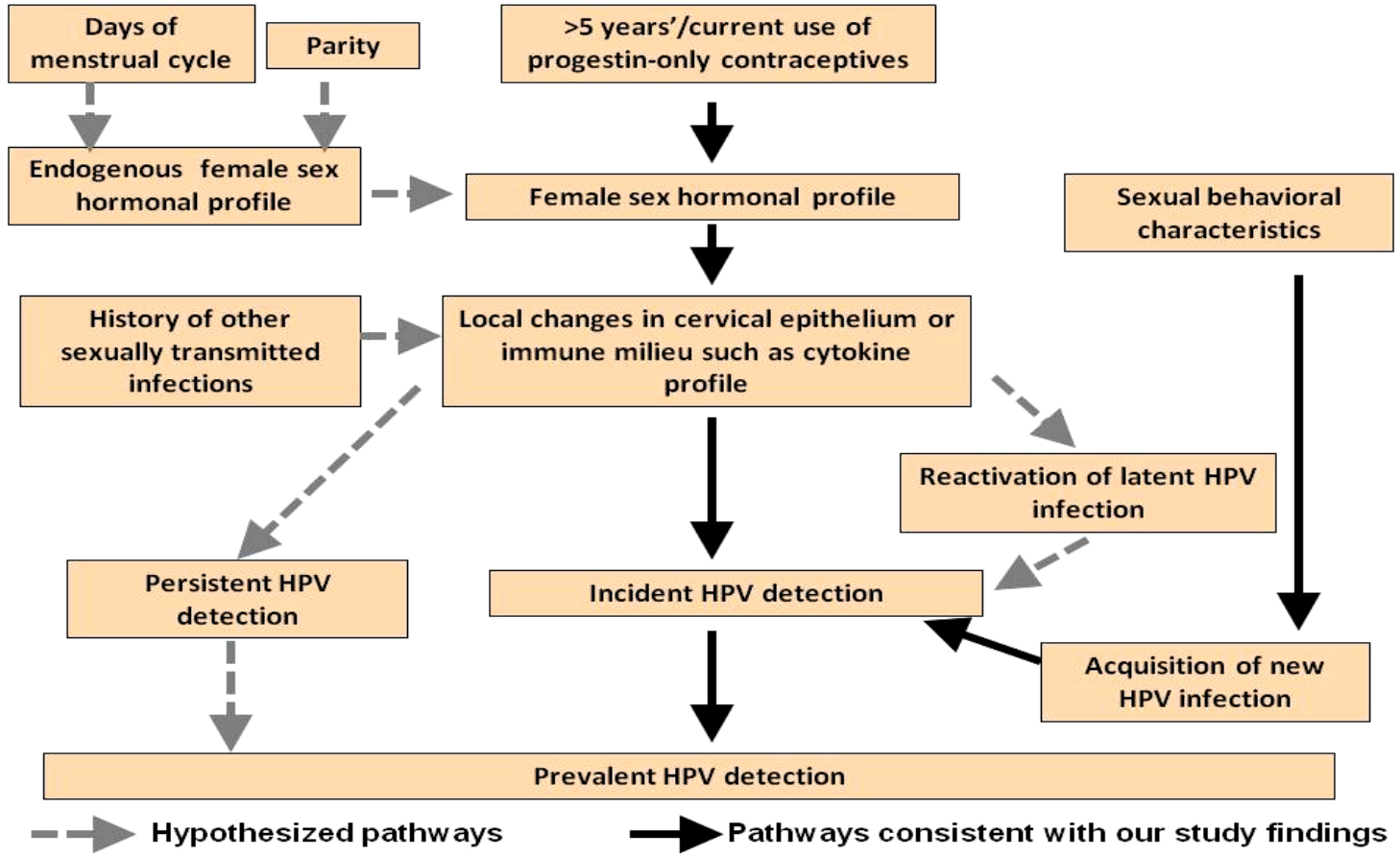

Figure 6.1. Conceptual model showing hypothesized relationships among HPV detection, progestin-only contraceptive use and other correlates of HPV detection in older women. ***Black solid arrows*****: Hypothesized pathways consistent with study findings in this dissertation research.** ***Gray dashed arrows*****: Hypothesized pathways suggested by findings reported in literature.**

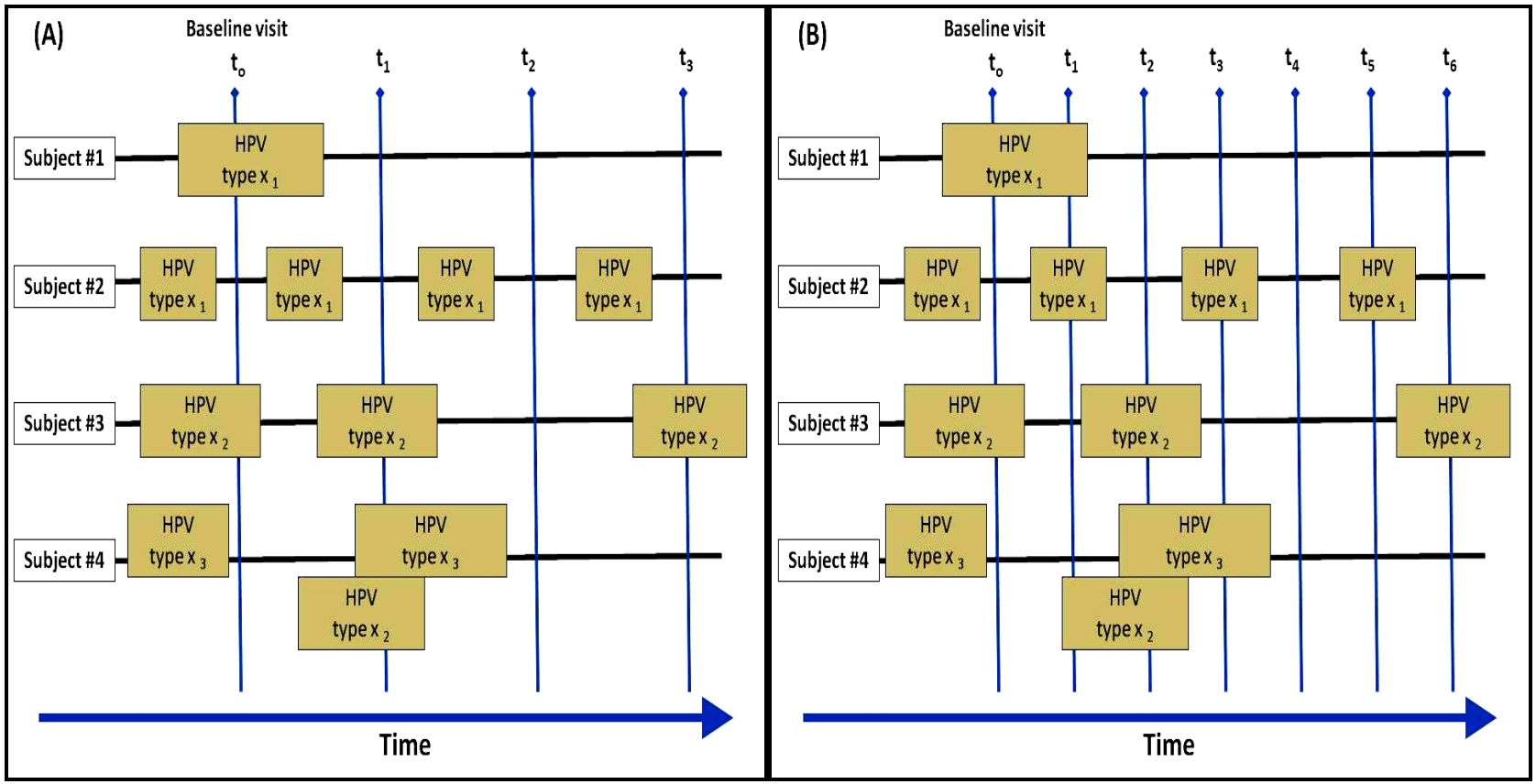

Figure 6.2. Schematic diagram illustrating left truncation and interval sampling bias in the context of different durations of HPV infection. A. Study design with less frequent interval sampling. B. Study design with more frequent interval sampling

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Figure 6.2. (A and B) Schematic diagram illustrating left truncation and interval sampling bias in the context of different durations of HPV infection.

Discrepancies in observed HPV status during follow-up by study design			
Hypothesized scenarios	**True HPV status over time**	**HPV status observed over time via study design A**	**HPV status observed over time via study design B**
Subject #1	• HPV-positive at study entry	• HPV-positive at t_0; infection clears before t_1 • No new detection after t_0	• HPV-positive at t_0; infection persists at t_1 and clears before t_2 • No new detection after t_1
Subject #2	• HPV-positive (HPV type x_1) before t_0 • 3 recurrent transient "reactivations" of HPV type x_1 over time	• HPV-negative at baseline and during follow-up	• New detection of HPV type x_1 at t_1 • 2 recurrent detections of HPV type x_1 at t_3, t_5
Subject #3	• HPV-positive (HPV type x_2)at t_0 • 2 recurrent transient "reactivations" of HPV type x_2 over time	• HPV-positive at t_0; infection persists at t_1 • 1 recurrent transient detection of HPV type x_2 over time at t_3	• HPV-positive at t_0; infection clears before t_1 • 2 recurrent transient detections of HPV type x_2 over time at t_2 and t_6
Subject #4	• HPV-positive (HPV type x_3) before t_0 • New detection of HPV type x_2, which later in time overlaps with recurrence of HPV type x_3	• HPV-negative at baseline • New co-detection of HPV type x_2 and type x_3 at t_1 • No persistence of detection is noted during follow-up	• HPV-negative at baseline • New detection of HPV type x_3 at t_2 that persists through t_3 • New detection of HPV type x_2 at t_1 that persists through t_2

CHAPTER 7:

Appendices

Aim 1

Appendix Table 3.1.

Association between HPV detection and total exposure to any progestin-only contraceptives, with never users of hormonal contraceptives as reference group (N=530)

Characteristics		Total N N(col %)	Positive for any HPV n=87 (16.4%) Any HPV+ n(row %)	Unadjusted PR (95% CI)	Adjusted PR[a] (95% CI)	Positive for HR-HPV n=34 (6.4%) HR-HPV+ n(row %)	Unadjusted PR (95% CI)	Adjusted PR[a] (95% CI)
Use of progestin-only contraceptives								
Total duration of use	**Years**							
• Never used any hormonal contraceptives		56 (10.6)	7 (12.5)	Ref	Ref	4 (7.1)	Ref	Ref
• Never used any progestin-only contraceptives but used other hormonal contraceptives		384 (72.5)	56 (14.6)	1.17 (0.56-2.43)	1.02 (0.50-2.10)	20 (5.2)	0.73 (0.26-2.06)	0.60 (0.21-1.75)
• Ever users of progestin-only contraceptives								
	≤5	72 (13.6)	15 (20.8)	1.67 (0.73-3.81)	1.19 (0.52-2.71)	5 (6.9)	0.97 (0.27-3.46)	0.84 (0.23-3.03)
	>5	16 (3.0)	8 (50.0)	**4.0 (1.71-9.35)**	**3.21 (1.37-7.52)**	4 (25.0)	3.5 (0.98-12.47)	2.73 (0.73-3.03)
• Unclear duration		2 (0.4)	1 (50.0)	4.0 (0.85-18.87)	2.09 (0.61-7.23)	1 (50.0)	**7.0 (1.31-37.51)**	3.56 (0.51-24.79)

[a] Adjusted for days from last menstrual period, number of lifetime sex partners, acquisition of new sex partner in last 6 months, marital status, time since last abnormal pap smear, history of other sexually transmitted diseases in the last 6 months.

Appendix Table 3.2.

Association between HPV detection and recency of any progestin-only hormonal contraceptive use, with never users of hormonal contraceptives as reference group (N=530)

Characteristics	Total N N(col %)	Positive for any HPV n=87 (16.4%) Any HPV+ n(row %)	Unadjusted PR (95% CI)	Adjusted PR[a] (95% CI)	Positive for HR-HPV n=34 (6.4%) HR-HPV+ n(row %)	Unadjusted PR (95% CI)	Adjusted PR[a] (95% CI)
Use of progestin-only contraceptives							
Last time of use							
• Never used any hormonal contraceptives	56 (10.6)	7 (12.5)	Ref	Ref	4 (7.1)	Ref	Ref
• Never used any progestin-only contraceptives but used other hormonal contraceptives	384 (72.5)	56 (14.6)	1.17 (0.56-2.43)	1.02 (0.50-2.09)	20 (5.2)	0.73 (0.26-2.06)	0.60 (0.20-1.76)
• Current user	47 (8.9)	13 (27.7)	2.21 (0.96-5.09)	1.60 (0.70-3.67)	6 (12.8)	1.79 (0.54-6.00)	1.57 (0.43-5.70)
• Past user	43 (8.1)	11 (25.6)	2.05 (0.87-4.84)	1.54 (0.66-3.60)	4 (9.3)	1.30 (0.34-4.92)	1.05 (0.30-3.63)

[a] Adjusted for days from last menstrual period, number of lifetime sex partners, acquisition of new sex partner in last 6 months, marital status, time since last abnormal pap smear, history of other sexually transmitted diseases in the last 6 months.

Appendix Table 3.3.

Association between HPV detection and total exposure to any oral contraceptives, with never users of hormonal contraceptives as reference group (N=530)

Characteristics		Total N N(col %)	Positive for any HPV n=87 (16.4%) Any HPV+ n(row %)	Unadjusted PR (95% CI)	Adjusted PR[a] (95% CI)	Positive for HR-HPV n=34 (6.4%) HR-HPV+ n(row %)	Unadjusted PR (95% CI)	Adjusted PR[a] (95% CI)
Use of oral contraceptives								
Total duration of use	**Years**							
• Never used any hormonal contraceptives		56 (10.6)	7 (12.5)	Ref	Ref	4 (7.1)	Ref	Ref
• Never used any oral contraceptives but used other hormonal contraceptives		39 (7.4)	14 (35.9)	**2.87 (1.28-6.46)**	1.91 (0.86-4.21)	6 (15.4)	2.15 (0.65-7.14)	1.52 (0.47-4.89)
• Ever users of oral contraceptives								
	≤5	211 (39.8)	32 (15.2)	1.21 (0.57-2.60)	1.01 (0.48-2.14)	14 (6.6)	0.93 (0.32-2.71)	0.82 (0.28-2.41)
	>5	197 (37.2)	30 (15.2)	1.22 (0.57-2.63)	1.12 (0.53-2.39)	7 (3.6)	0.50 (0.15-1.64)	0.37 (0.11-1.19)
• Unclear duration		27 (5.1)	4 (14.8)	1.19 (0.38-3.71)	0.89 (0.32-2.52)	3 (11.1)	1.56 (0.37-6.48)	1.08 (0.26-4.51)

[a] Adjusted for days from last menstrual period, number of lifetime sex partners, acquisition of new sex partner in last 6 months, marital status, time since last abnormal pap smear, history of other sexually transmitted diseases in the last 6 months.

Appendix Table 3.4.

Association between HPV detection and recency of any oral contraceptive use, with never users of hormonal contraceptives as reference group (N=530)

Characteristics	Total N N(col %)	Positive for any HPV n=87 (16.4%) Any HPV+ n(row %)	Unadjusted PR (95% CI)	Adjusted PR[a] (95% CI)	Positive for HR-HPV n=34 (6.4%) HR-HPV+ n(row %)	Unadjusted PR (95% CI)	Adjusted PR[a] (95% CI)
Use of oral contraceptives							
Last time of use							
• Never used any hormonal contraceptives	56 (10.6)	7 (12.5)	Ref	Ref	4 (7.1)	4 (7.1)	Ref
• Never used any oral contraceptives but used other hormonal contraceptives	39 (7.4)	14 (35.9)	**2.87 (1.28-6.46)**	1.91 (0.86-4.21)	20 (5.2)	6 (15.4)	1.55 (0.49-4.i97)
• Current user	63 (11.9)	10 (15.9)	1.27 (0.52-3.11)	0.97 (0.40-2.36)	6 (12.8)	4 (6.4)	0.56 (0.13-2.40)
• Past user	372 (70.2)	56 (15.1)	1.20 (0.58-2.51)	1.06 (0.52-2.17)	4 (9.3)	20 (5.40	0.67 (0.23-1.94)

[a] Adjusted for days from last menstrual period, number of lifetime sex partners, acquisition of new sex partner in last 6 months, marital status, time since last abnormal pap smear, history of other sexually transmitted diseases in the last 6 months.

Appendix Table 3.5.

Association between HPV detection and total exposure to combined oral contraceptive use (N=530)

Characteristics		Total N N(col %)	Positive for any HPV n=87 (16.4%) Any HPV+ n(row %)	Unadjusted PR (95% CI)	Adjusted PR[a] (95% CI)	Positive for HR-HPV n=34 (6.4%) HR-HPV+ n(row %)	Unadjusted PR (95% CI)	Adjusted PR[a] (95% CI)
Use of combined oral contraceptives								
Total duration of use	**Years**							
• Never used any hormonal contraceptives		56 (10.6)	7 (12.5)	Ref	Ref	4 (7.1)	Ref	Ref
• Never used any combined oral contraceptives but used other hormonal contraceptives		34 (6.4)	13 (38.2)	**3.06 (1.35-6.91)**	1.81 (0.80-4.10)	5 (14.7)	1.06 (0.59-7.15)	1.50 (0.42-5.32)
• Ever users of combined oral contraceptives								
	≤5	101 (19.1)	11 (10.9)	0.87 (0.36-2.12)	0.74 (0.31-1.78)	5 (5.0)	0.69 (0.19-2.48)	0.62 (0.17-2.28)
	>5	86 (16.2)	13 (15.1)	1.21 (0.51-2.85)	1.05 (0.45-2.45)	3 (3.5)	0.49 (0.11-2.10)	0.35 (0.08-1.51)
• Reported use of "pills" without specifying medication brand names		253 (47.7)	43 (17.0)	1.36 (0.65-2.86)	1.20 (0.58-2.48)	17 (6.7)	0.94 (0.33-2.70)	0.77 (0.26-2.24)

[a] Adjusted for days from last menstrual period, number of lifetime sex partners, acquisition of new sex partner in last 6 months, marital status, time since last abnormal pap smear, history of other sexually transmitted diseases in the last 6 months.

Appendix Table 3.6.

Association between HPV detection and recency of combined oral contraceptive use (N=530)

Characteristics	Total N N(col %)	Positive for any HPV n=87 (16.4%) Any HPV+ n(row %)	Unadjusted PR (95% CI)	Adjusted PR[a] (95% CI)	Positive for HR-HPV n=34 (6.4%) HR-HPV+ n(row %)	Unadjusted PR (95% CI)	Adjusted PR[a] (95% CI)
Use of combined oral contraceptives							
Last time of use							
• Never used any hormonal contraceptives	56 (10.6)	7 (12.5)	Ref	Ref	4 (7.1)	Ref	Ref
• Never used any combined oral contraceptives but used other hormonal contraceptives	34 (6.4)	13 (39.4)	**3.15 (1.40-7.10)**	1.83 (0.81-4.13)	5 (15.2)	2.12 (0.61-7.36)	1.50 (0.42-5.40)
• Current user	46 (8.7)	6 (13.0)	1.04 (0.38-2.89)	0.90 (0.33-2.46)	3 (6.5)	0.91 (0.21-3.88)	0.76 (0.16-3.51)
• ≤5 years ago	32 (6.0)	2 (6.3)	0.50 (0.11-2.27)	0.33 (0.07-1.54)	1 (3.1)	0.44 (0.05-3.76)	0.26 (0.03-2.45)
• 6-10 years ago	29 (5.5)	6 (20.7)	1.66 (0.61-4.48)	1.46 (0.54-3.95)	1 (3.5)	0.48 (0.06-4.13)	0.42 (0.07-2.48)
• >10 years ago	81 (15.3)	10 (12.1)	0.96 (0.39-2.38)	0.93 (0.38-2.25)	3 (3.6)	0.51 (0.12-2.18)	0.46 (0.10-2.04)
• Use of "the pills" without specifying medication brand names	253 (47.7)	43 (17.1)	1.37 (0.65-2.89)	1.20 (0.58-2.50)	17 (6.8)	0.95 (0.33-2.71)	0.78 (0.27-2.26)

[a] Adjusted for days from last menstrual period, number of lifetime sex partners, acquisition of new sex partner in last 6 months, marital status, time since last abnormal pap smear, history of other sexually transmitted diseases in the last 6 months.

Aim 2

Appendix Table 4.1. Baseline characteristics of study population who had at least 6-month follow-up at the time of analysis

Characteristics at baseline	Total at baseline N=530 (col %)[a]	Population with at least 6-month follow-up N=383 (72.3%) (col %)	Population not yet having any follow-up visit N=147 (27.7%) (col %)	P
Baseline status of any HPV				0.12
Negative	443 (83.6)	326 (85.1)	117 (79.6)	
Positive	87 (16.4)	57 (14.9)	30 (20.4)	
Baseline status of any HR-HPV				**0.01**
Negative	496 (93.6)	365 (95.3)	1131 (89.1)	
Positive	34 (6.4)	18 (4.7)	16 (10.9)	
Demographic characteristics				
Age (years)				
35-39	126 (23.8)	98 (25.6)	28 (19.1)	**0.004**
40-44	148 (27.9)	103 (26.9)	45 (30.6)	
45-49	157 (29.6)	119 (31.1)	38 (25.9)	
≥50	99 (18.7)	63 (17.5)	36 (24.5)	
Race				0.58
Caucasian	392 (74.0)	288 (75.2)	104 (70.8)	
African American	99 (18.7)	68 (17.8)	31 (21.1)	
Others[b]	39 (7.4)	27 (7.1)	12 (8.2)	
Education status				**0.03**
High school	84 (15.9)	56 (14.6)	28 (19.1)	
Post-high school	122 (23.0)	78 (20.4)	44 (29.9)	
College graduate	166 (31.3)	129 (33.7)	37 (25.2)	
Post-college	158 (29.8)	120 (31.3)	38 (25.9)	
Marital status				0.32
Married	339 (64.0)	252 (65.8)	87 (59.2)	
Single	107 (20.2)	75 (19.6)	32 (21.8)	
Divorced/separated/widowed	84 (15.9)	56 (14.6)	28 (19.1)	
Current smoker				**0.03**
No	475 (89.6)	350 (91.4)	125 (85.0)	

Yes	55 (10.4)	33 (8.6)	22 (15.0)	
Parity				0.58
0 or 1	170 (32.1)	124 (32.5)	46 (31.3)	
2	124 (23.4)	85 (22.3)	39 (26.5)	
>2	235 (44.4)	173 (45.3)	62 (42.2)	
Sexual behavioral characteristics				
Lifetime number of sexual partners				**0.05**
≤2	59 (11.1)	51 (13.3)	8 (5.4)	
3-4	134 (25.3)	99 (25.9)	35 (23.8)	
5-6	215 (40.6)	150 (39.2)	65 (44.2)	
>10	122 (23.0)	83 (21.7)	39 (26.5)	
Sexual activity in last 6 months				0.46
No sex	86 (16.2)	58 (15.1)	28 (19.1)	
Have sex but no new sexual partner	429 (80.9)	313 (81.7)	116 (78.9)	
Have sex with new sexual partner	15 (2.8)	12 (3.1)	3 (2.0)	
Last menstrual period (days)				**0.001**
1-14	227 (42.8)	178 (46.5)	49 (33.3)	
15-28	174 (32.8)	123 (32.1)	51 (34.7)	
28-40	34 (6.4)	28 (7.3)	6 (4.1)	
40-365	95 (17.9)	54 (14.1)	41 (27.9)	
Ever undergone colposcopy				0.21
No	425 (80.2)	302 (78.9)	123 (83.7)	
Yes	105 (19.8)	81 (21.2)	24 (16.3)	
Ever undergone treatment for abnormal pap smear				0.57
No	430 (81.1)	33 (81.3)	117 (79.6)	
Yes	100 (18.9)	70 (18.3)	30 (20.4)	
Time since last abnormal pap smear				0.80
Never had abnormal pap smear	289 (54.5)	210 (54.8)	79 (53.7)	
<1 year ago	11 (2.1)	9 (2.4)	2 (1.4)	
2-5 years ago	59 (11.1)	44 (11.5)	15 (10.2)	
≥6 years ago	171 (32.3)	120 (31.3)	51 (34.7)	
Diagnosed with other sexually transmitted infections in the past 6 months [c]				0.31

No		517 (94.5)	372 (97.1)	145 (98.6)	
Yes		13 (5.5)	11 (2.9)	2 (1.4)	
Hormonal contraceptives:					
Total lifetime exposure					
Duration of use by recency	**Years**				0.78
Never used any hormonal contraceptives		56 (10.6)	42 (11.0)	14 (9.5)	
Current	≤5	82 (15.5)	58 (15.1)	24 (16.3)	
	>5	30 (5.9)	24 (6.3)	7 (4.8)	
Former	≤5	179 (33.8)	134 (35.0)	45 (30.6)	
	>5	161 (30.4)	111 (29.0)	50 (34.0)	
Unclear duration		22 (4.0)	14 (3.7)	7 (4.8)	
Time since last use					0.78
Never user		56 (10.6)	42 (11.0)	14 (9.5)	
Current user		112 (21.1)	81 (21.2)	31 (21.1)	
Last use ≤ 5 years ago		74 (14.0)	57 (14.9)	17 (11.6)	
Last use 6-10 years ago		63 (11.9)	46 (12.0)	17 (11.6)	
Last use >10 years ago		225 (42.5)	157 (41.0)	68 (46.3)	
Use of any progestin-only contraceptives					
Duration of use	**Years**				0.89
Never used any progestin-only contraceptives		440 (83.0)	319 (83.3)	121 (82.3)	
	≤5	72 (13.6)	51 (13.3)	21 (14.3)	
	>5	16 (2.8)	12 (3.1)	4 (2.7)	
Unclear		2 (0.4)	1 (0.3)	1 (0.7)	
Time since last use					0.11
Never used any progestin-only contraceptives		440 (83.0)	319 (83.3)	121 (82.3)	
Current user		47 (8.9)	29 (7.6)	18 (12.2)	
Past user		43 (8.1)	35 (9.1)	8 (5.4)	
Use of oral contraceptives					
Duration of use	**Years**				1.00

Never used any oral contraceptives		95 (17.9)	69 (18.0)	26 (17.7)	
	≤5	211 (39.8)	153 (40.0)	58 (39.5)	
	>5	197 (37.2)	142 (37.1)	55 (37.4)	
Unclear duration		27 (5.1)	19 (5.0)	8 (5.44)	
Time since last use					0.39
Never used any oral contraceptives		95 (17.9)	69 (18.0)	26 (17.7)	
Current user		63 (11.9)	50 (13.1)	13 (8.8)	
Past user		372 (70.2)	264 (68.9)	108 (73.5)	

[a.] Percentages or numbers in categories may not add up to 100% or the total number due to missing data.
[b.] Other categories include American Indian (n=2), Pacific Islander (n=2), Asian (n=16), unidentified (n=9).
[c.] Other sexually transmitted infections included genital chlamydia, gonorrhea, herpes, syphilis, trichomoniasis, chancroid, warts.

Appendix Table 4.2. Baseline characteristics of visits with persistent HPV detection during follow-up (Total number of positive HPV detections that had 6 month-follow-up: 179)

Characteristics at baseline	Total number of positive HPV detections that had 6 month-follow-up N=179 (col%)	Any HPV detection with persistence at 6 months N=110(61.5%) (row %)	P
Demographic characteristics			
Age at baseline (years)			0.38
35-39	61 (34.1)	42 (68.9)	
40-44	31 (17.3)	18 (58.1)	
45-49	59 (33.0)	36 (61.0)	
≥50	28 (15.6)	14 (51.9)	
Race			
Caucasian	137 (76.5)	85 (62.0)	0.91
African American	31 917.3)	18 (58.1)	
Others [c]	11 (6.2)	7 (63.60	
Marital status			0.83
Married	68 (38.0)	40 (58.8)	
Single	47 (26.3)	29 (61.7)	
Divorced/separated/widowed	64 (35.8)	41 (64.1)	
Smoker in the past 6 months			0.40
No	167 (93.3)	104 (62.3)	
Yes	12 (6.7)	6 (50.0)	
Parity at baseline			0.63
0 or 1	95 (53.1)	59 (62.1)	
2	28 (15.6)	19 (67.9)	
>2	56 (31.3)	32 (57.1)	
Sexual behavioral characteristics			
Lifetime number of sexual partners			0.95
<5	32 (17.9)	19 (59.4)	
5-10	96 (53.60	60 (62.5)	
>10	51 (28.5)	31 (60.8)	

Sexual activity in last 6 months			0.81
No sex	20 (11.2)	11 (55.0)	
Have sex but no new sexual partner	137 (76.5)	85 (62.0)	
Have sex with new sexual partner	22 (12.3)	14 (63.6)	
Last menstrual period (days)			0.29
1-14	57 (31.8)	30 (52.6)	
15-28	76 (42.5)	48 (63.2)	
28-40	10 (5.6)	6 (60.0)	
40-365	36 (20.1)	26 (72.2)	
Ever undergone colposcopy			0.60
No	131 (73.2)	82 (62.6)	
Yes	48 (26.8)	28 (58.3)	
Time since last abnormal pap smear at baseline			0.16
Never had abnormal pap smear	92 (51.4)	63 (68.5)	
<1 year ago	10 (5.6)	4 (40.0)	
2-5 years ago	23 (12.9)	14 (60.9)	
≥6 years ago	54 (30.2)	29 (53.7)	
Diagnosed with other sexually transmitted infections in the past 6 months*			0.08
No	170 (95.0)	102 (60.0)	
Yes	9 (5.0)	8 (88.9)	

* Other sexually transmitted infections included genital chlamydia, gonorrhea, herpes, syphilis, trichomoniasis, chancroid, warts.

Appendix Table 4.3. Baseline hormonal contraceptive use and persistent HPV detection at 6 months during follow-up (Total number of positive HPV detections that had 6 month-follow-up: 179)

Characteristics at baseline		Total number of positive HPV detections that had 6 month-follow-up N=179 (col %)	Any HPV detection with persistence at 6 months N=110(61.5%) (row %)	P
Hormonal contraceptives:				
Total lifetime exposure				
Duration of use by recency	**Years**			0.32
Never used any hormonal contraceptives		19 (10.6)	12 (63.2)	
	≤5	101 (56.4)	66 (65.4)	
	>5	54 (30.2)	28 (51.9)	
Unclear duration		5 (2.8)	4 (80.0)	
Time since last use				0.65
Never user		19 (10.6)	12 (63.2)	
Current user		50 (27.9)	28 (56.0)	
Past user		110 (61.5)	70 (63.6)	
Use of any progestin-only contraceptives				
Duration of use	**Years**			0.58
Never used any progestin-only contraceptives		122 (68.2)	79 (64.8)	
Ever users				
	≤5	45 (25.1)	24 (53.3)	
	>5	9 (5.0)	5 (55.6)	
Unclear		3 (1.7)	2 (66.7)	
Time since last use				0.13
Never used any progestin-only contraceptives		122 (68.2)	79 (64.8)	

Current user		28 (15.6)	18 (64.3)	
Past users		29 (16.2)	13 (44.8)	
Use of any oral contraceptives				
				0.14
Duration of use by recency	**Years**			
Never used any oral contraceptives		45 (25.1)	26 (57.8)	
Ever users				
	≤5	61 (34.1)	42 (68.9)	
	>5	65 (36.3)	35 (53.9)	
Unclear duration		8 (4.5)	7 (87..5)	
Time since last use				0.26
Never used any oral contraceptives		45 (25.1)	26 (57.8)	
Current user		21 (11.7)	10 (47.6)	
Past user		113 (63.1)	74 (65.5)	

Aim 3

Appendix Table 5.1. Distribution of cytokine measurements that were outside detection range

	Protein-adjusted cytokine levels (pg cytokine/mg protein) Total samples (N=210)		Outside detection range (%)			
Cytokine	Median	IQR	Total samples (n=210)	Current HC users (n=105)	Non-current HC users (n=105)	p*
IL1b	38.75	89.53	10.4	8.9	11.9	0.49
IL1ra	6724.67	11334.13	81.7	82.2	81.2	0.86
IL2	1.90	2.10	2.5	4	1	0.17
IL4	0.31	0.22	0	0	0	na
IL5	0.65	0.74	59.9	66.3	53.5	0.06
IL6	23.84	70.75	0.5	0	1	0.32
IL7	2.89	3.01	2	3	1	0.31
IL8	217.61	227.41	54.5	56.4	52.5	0.57
IL9	5.13	5.55	1	2	0	0.16
IL10	6.33	6.99	0	0	0	na
IL12	28.74	32.95	0	0	0	na
IL13	4.01	3.85	2	3	1	0.31
IL15	2.02	3.72	16.8	18.1	14.9	0.45
IL17	7.15	6.64	0	0	0	na
EOTAXIN	5.61	11.58	33.7	31.7	35.6	0.55
GCSF	398.70	636.72	21.8	24.8	18.8	0.89
GMCSF	161.58	197.49	0.5	1	0	0.32
IFN-γ	25.28	17.99	0	0	0	na
IP10	517.17	1191.11	3	3	3	1
MCP1	37.72	76.66	4	5.9	2	0.15
MIP1a	1.97	2.80	1.5	1	2	0.56
MIP1b	39.81	67.28	6.9	6.9	6.9	1
RANTES	7.33	15.26	5.5	5	5.9	0.76
TNF-α	5.26	5.37	0	0	0	na
VEGF	348.32	427.62	3.5	2.0	5.0	0.25
FGF	20.06	32.36	0.5	0	1	0.32
PDGF	46.94	50.61	0	0	0	na

*McNemar's test

Appendix Table 5.2. Current hormonal contraceptive use by type among subjects included for cervical cytokine measurement

	Total number of cases included for study (N=105) n (%)
Use of any hormonal contraceptive	105 (100)
Combined oral contraceptive (COC)	41 (39.0)
"Birth control pills"/"pills"	13 (12.4)
Progestin-only contraceptives Mirena Oral pills DMPA	 23 (21.9) 21 (20.0) 6 (5.7)
Nuva ring	3 (2.9)
Current use of dermal patch	1 (1.0)
Overlapping use of COC and mirena	1 (1.0)
Unclear route	2 (1.9)

Curriculum Vitae

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EDUCATION

05/2009 – 07/2015 (Defended in 07/2015) **Ph.D., Epidemiology**
Johns Hopkins Bloomberg School of Public Health, Baltimore MD
Advisor: *Patti Gravitt Ph.D., M.S.*
Dissertation topic*: Association of hormonal contraceptive use with human papillomavirus (HPV) detection in pre- and perimenopausal women in the U.S.*

07/2008 – 05/2009 **M.P.H.**
Johns Hopkins Bloomberg School of Public Health, Baltimore MD

09/2003 – 05/2011 **M.D.**
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08/1999 – 05/2003 **B.S., double majors in Molecular Environmental Biology and Nutritional Sciences**
University of California, Berkeley CA

POSTGRADUATE TRAINING

07/2012 – present **Anatomic and Clinical Pathology Resident**
Department of Pathology, Brigham and Women's Hospital, Boston MA

MEDICAL LICENSURE

07/2012 – present **Board of Registration in Medicine, Commonwealth of Massachusetts, MA**

HONORS AND AWARDS

07/2011	**Travel Award** International Society for STD Research (ISSTDR)
05/2011	**The Harvey M. Meyerhoff Fellowship in Cancer Prevention** Johns Hopkins Bloomberg School of Public Health
06/2009	**Excellence in Medical Student Research Award** Johns Hopkins University School of Medicine
05/2009	**Inductee, Delta Omega Honorary Society in Public Health, Alpha Chapter** Johns Hopkins Bloomberg School of Public Health
07/2008	**The Anonymous Endowed Scholarship Fund** Johns Hopkins Bloomberg School of Public Health
05/2007	**Second Place Award, Clinical Vignette Poster Competition for Medical Students** American College of Physicians, 25th Annual Maryland Associates Meeting
06/2006 – 05/2007	**Howard Hughes Research Training Fellowship** Howard Hughes Medical Institute
06/2004 – 08/2004	**Dean's Summer Research Fund** Johns Hopkins University School of Medicine
05/2003	**Inductee, Phi Beta Kappa** University of California, Berkeley
05/2002	**Molecular Environmental Biology Departmental Citation** University of California, Berkeley
05/2001	**Biology Fellow Award** University of California, Berkeley
05/2001	**Edith G. Stokstad Memorial Scholarship** University of California, Berkeley
10/2000	**Edward Frank Kraft Scholarship Prize** University of California, Berkeley
1999-2003	**Dean's Honor List** University of California, Berkeley

PUBLICATIONS

Peer-reviewed articles:

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Soong TR, Burke AE, Viscidi R, Silver M, Chang K, Gravitt PE. Evaluating the association of hormonal contraceptive use with HPV detection in pre- and perimenopausal women in the U.S. *28th International Papillomavirus Conference. San Juan, Puerto Rico. Poster presentation (December 3, 2012)*

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Soong TR, Gravitt PE, Gupta SB, Liaw K, Kim K, Tadesse A, Phongnarisorn C, Wootipoom V, Yuenyao P, Vipupinyo C, Sriplienchan S, Celentano DD. Genital chlamydia and gonorrhea are independent predictors of HPV prevalence. *North American Congress of Epidemiology, Montreal, Canada. Poster presentation. (June 21, 2011)*

Soong TR, Jung, JJ, Rothman RE, Gabor KD, Burah A, Shahan J, Hsieh Y-H. Inadequate HIV care is associated with increased emergency department utilization and hospitalizations: A prospective study in HIV-positive patients. *Society for Academic Emergency Medicine 2010 Annual Meeting, Phoenix, AZ. Poster presentation. (June 3, 2010)*

Soong TR, Pathak AP, Asano H, Fox-Talbot K, Baldwin WM 3rd. Lymphatic injury and regeneration patterns are associated with alloimmune responses in cardiac allografts. *American Transplant Congress, Toronto, Canada. Poster presentation. (May 31, 2008)*

Soong TR, Barouch LA, Champion HC, Wigley FM, Halushka MK. New clinical and ultrastructural findings in hydroxychloroquine-induced cardiomyopathy – a report of two cases. *American College of Physicians 25th Annual Maryland Associates Meeting, Baltimore, MD. Poster presentation (May 17, 2007) (Poster was also selected for presentation at national meeting of Internal Medicine 2008, Washington DC, May 15, 2008)*

TEACHING EXPERIENCE

05/2015	**Course Instructor** Microbiology Clinical Pathological Correlation Conferences of Immunology, Infectious Disease, and Pathology (IMP) Course Harvard Medical School, Boston MD
2014-2015	**Short Course Instructor** Practical Statistics for Pathologists: Case-Based Instruction with Methods United States and Canadian Academy of Pathology (USCAP) 2014 Annual Meeting, San Diego CA (03/26/2015) United States and Canadian Academy of Pathology (USCAP) 2015 Annual Meeting, Boston MA (03/05/2014)
08/2009 – 03/2012	**Teaching Assistant** PH 340.751-752 (Epidemiologic Methods) PH 140.621-623 (Statistical Methods in Public Health) Johns Hopkins Bloomberg School of Public Health, Baltimore MD
12/2008 – 01/2009	**Student Instructor,** gross anatomy sessions for first-year medical students, Johns Hopkins University School of Medicine, Baltimore MD
06/2004 – 08/2004	**Tutor,** MCAT preparatory courses Princeton Review, Baltimore MD
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MEMBERSHIP IN PROFESSIONAL SOCIETY

01/2013 – present	**United States and Canadian Academy of Pathology**

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